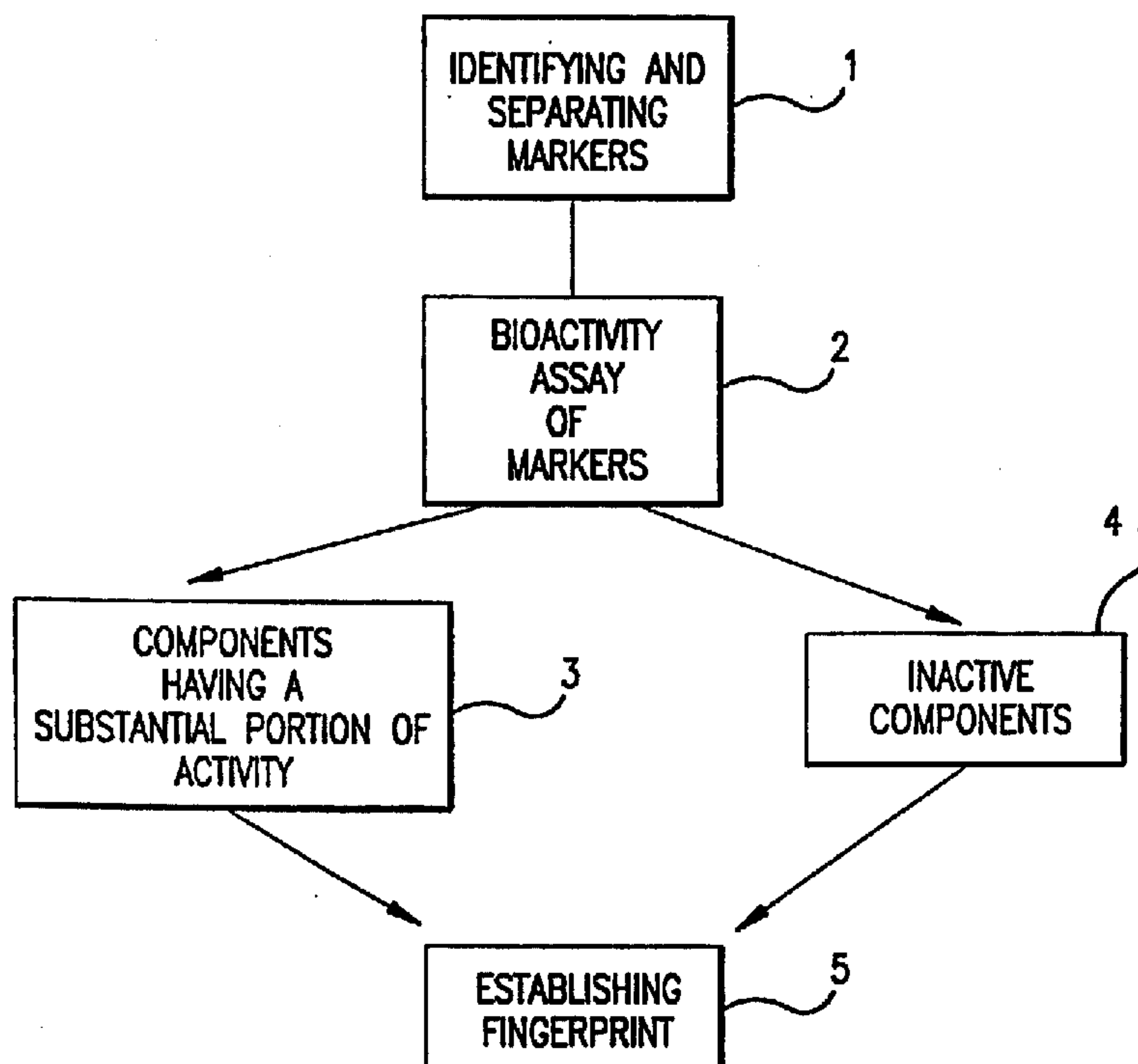




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(54) **AIL DE QUALITE PHARMACEUTIQUE**
(54) **PHARMACEUTICAL GRADE GARLIC**



(57) La présente invention a trait de manière générale à des matières d'ail et à des procédés de transformation de ces matières en formes utiles d'un point de vue médicinal et pharmaceutiquement acceptables. Plus particulièrement, l'invention a trait à l'utilisation d'empreintes de composition et d'activité dans le traitement de matières d'ail pour produire des médicaments qui remplissent les conditions requises pour constituer des compositions de qualité pharmaceutique convenant pour un usage clinique ou vétérinaire en vue de traiter et/ou soulager des maladies, des affections ou des états pathologiques.

(57) The present invention relates generally to garlic materials and methods for making such materials in medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of garlic materials to produce drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical or veterinary settings to treat and/or ameliorate diseases, disorders or conditions.

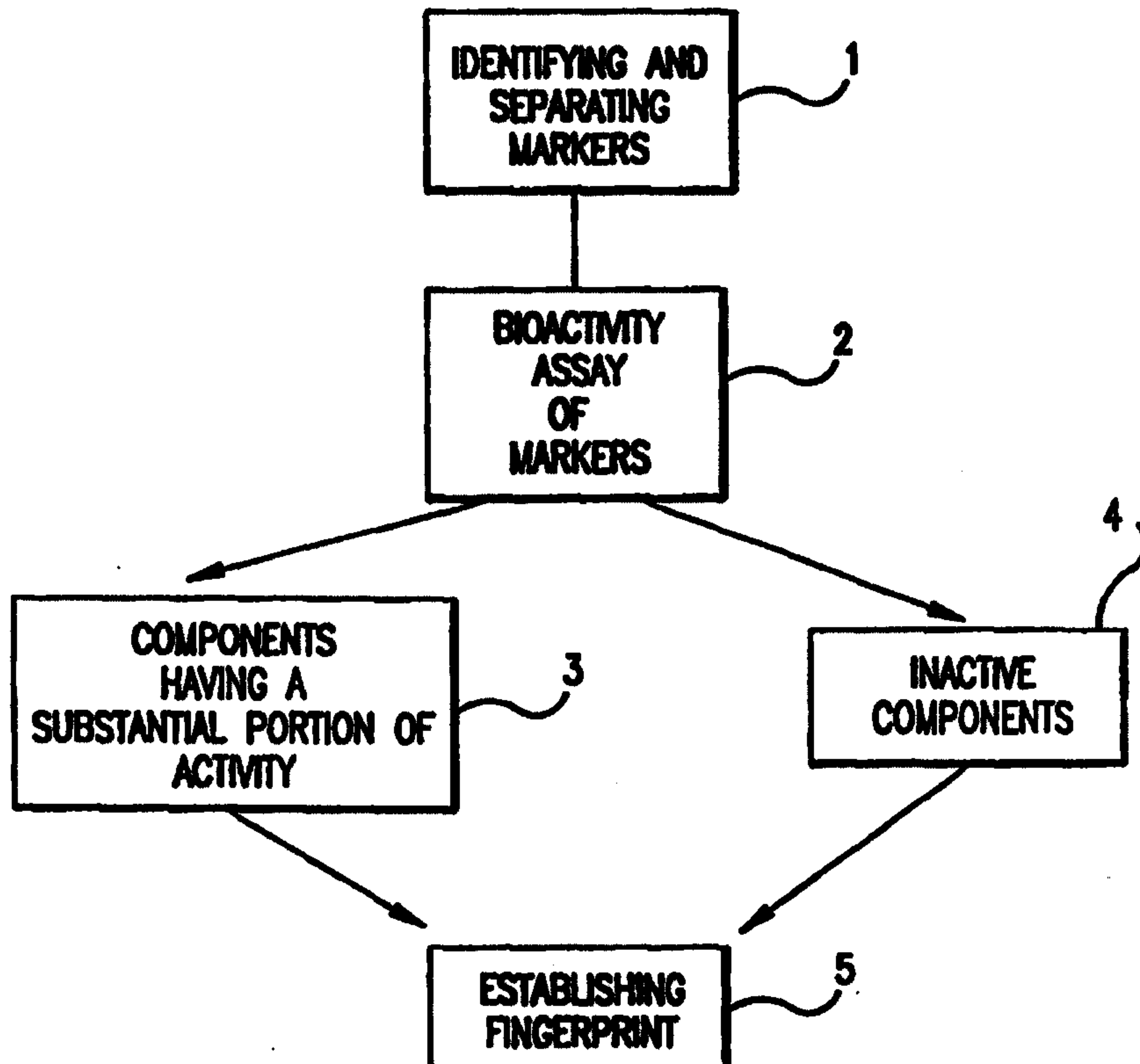
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(21) International Application Number: PCT/US98/22508 (22) International Filing Date: 23 October 1998 (23.10.98) (30) Priority Data: 08/956,718 23 October 1997 (23.10.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/956,718 (CIP) Filed on 23 October 1997 (23.10.97) (71) Applicants (for all designated States except US): PHARMAPRINT, INC. [US/US]; Suite 1900, 4 Park Plaza, Irvine, CA 92614 (US). UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; University Park, Los Angeles, CA 90089 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KHWAJA, Tasneem, A. [US/US]; 7 Monterey Circle, Corona Del Mar, CA 92625 (US). FRIEDMAN, Elliot, P. [US/US]; 2160 Veloz, Montecito, CA 93108 (US).	(74) Agents: BALDWIN, Geraldine, F. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: PHARMACEUTICAL GRADE GARLIC**(57) Abstract**

The present invention relates generally to garlic materials and methods for making such materials in medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of garlic materials to produce drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical or veterinary settings to treat and/or ameliorate diseases, disorders or conditions.



PHARMACEUTICAL GRADE GARLIC

This is a continuation-in-part of co-pending U.S. Serial No. 08/956,718, filed October 23, 1997, which is a continuation-in-part of co-pending U.S. Serial No. 5 08/838,198, filed on April 15, 1997, the entire disclosure of which is incorporated herein by reference, which is a continuation-in-part of co-pending U.S. Serial No. 08/632,273, filed on April 15, 1996, which is a continuation-in-part of U.S. Serial No. 08/421,993, filed on April 14, 1995 abandoned in favor of U.S. Serial No. 08/774,550, filed 10 February 4, 1997.

1. FIELD OF THE INVENTION

The present invention relates generally to botanical materials and methods for transforming such materials into medicinally useful and pharmaceutically acceptable forms. 15 More particularly, the present invention relates to the use of compositional and bioactivity fingerprints in the processing of botanical materials of *Allium sativum* L. (garlic) to produce botanical drugs which qualify as pharmaceutical grade compositions which are suitable for use 20 in clinical settings to treat and/or ameliorate diseases, disorders and/or conditions.

2. BACKGROUND OF THE INVENTION

Pharmaceutical manufacturing is based on control over the composition and bioactivity for each manufactured batch. 25 This standardization and control provides reproducible material in the predictable and consistent treatment of patients. Herbal medicines, produced from botanical materials, have presented a unique problem for manufacturers desiring the control, reproducibility, and standardization that are required of pharmaceuticals. This problem is 30 primarily due to the plurality of components contained in an herbal medicine and the large variation in composition and

potency due to the growing, harvesting and processing conditions of raw materials.

Plants have been, and continue to be, the source of a wide variety of medicinal compounds. For centuries, various forms of botanically derived materials have been used to
5 treat countless different ailments. The botanical materials have typically been in the form of powders made from one or more plants or plant parts or extracts derived from whole plants or selected plant parts. These powders and extracts are, for the most part, complex mixtures of both biologically active and biologically inactive compounds.

10 Although plant powders and extracts have been used widely for medicinal purposes, there are a number of problems associated with the use of such medicaments. For example, the complex chemical nature of the botanical materials makes it difficult to use the botanical materials in any type of controlled and predictable manner. The potential variations
15 in the chemical composition of different batches of material obtained from different plant harvests makes such materials unsuitable for use in clinical situations.

On a positive note, the complex groupings of bioactive components typically found in botanical materials presents the potential for synergistic or additive bioactivity
20 profiles. However, these potential increases in medicinal effectiveness have not been predictable due to the unknown nature of these complex materials.

The above problems associated with the inherent chemical complexity of botanical medicaments has resulted in a great deal of effort being directed to the separation and isolation
25 of the biologically active components from numerous medicinally important botanical materials. This area of endeavor has expanded rapidly in conjunction with the many improvements in chemical separation and analysis technology. Once isolated and purified, the various active components may be used in clinical settings to establish the medicinal
30 effectiveness of a specific component. Separation and purification of individual components from botanical

materials is the cornerstone of this type of drug development procedure. Once purified, the suspected active component is typically mixed with a pharmaceutically acceptable carrier and subjected to further studies in laboratory animals and eventual clinical trials in humans. Upon proof of clinical
5 efficacy, these types of drugs are considered to be pharmaceutical grade because they contain a single, or at most a small number of, well-characterized compounds which are present in known quantities.

Pharmaceutical grade drugs are advantageous in that they allow careful tracking of the effects of individual compounds
10 in treatment protocols. Further, the dosage of the drug can be carefully controlled to provide relatively predictable medicinal action. A disadvantage of the relative purity of such pharmaceutical grade drugs is that the potential for complex and synergistic biological activity provided by naturally occurring plant materials is reduced because of the
15 isolation of the drug from its natural environment. The study of isolated products may also represent artifacts produced by breakdown of sensitive biological/botanical complexes. The potential benefit provided by such synergistic activity is believed by many industry experts to be outweighed by the clinical risks associated with the use
20 of complex plant materials which are not well characterized or controlled in a clinical setting.

Although isolation and purification of single compounds from plant materials has been a popular form of drug research and development, there has also been interest in studying complex botanical extracts to characterize their medicinal
25 qualities. Many complex plant materials and extracts exist which have potent, but relatively unpredictable, medicinal properties. These materials are, for the most part, useless in a clinical setting because of the inherent risks involved with treating patients with poorly characterized materials which have no established batch consistency and which may
30 differ widely in composition. Accordingly, there is a need to provide methods for standardizing such complex botanical

materials so that they may be used more effectively in clinical research and patient treatments.

2.1. GARLIC *Allium sativum* L. (Family Liliaceae)

5 Garlic as either the dried or fresh bulbs of *Allium sativum* L. of the lily family has been cultivated throughout the world for more than 5,000 years. An essential oil from garlic, obtained by steam distillation of the crushed fresh bulbs is also available.

10 Garlic has been widely consumed as a food and medicine since the times of the Egyptian pharaohs and the earliest Chinese dynasties (Foster, 1991, Garlic: *Allium sativum*, American Botanical Council: Austin, Texas, p. 1-7). Garlic and onion (*Allium cepa*) have both been used for millennia to treat cardiovascular and other disorders. They, their
15 extracts, and compounds isolated from them, have been tested for effects on hyperlipidemia, hypertension, hyperglycemia, platelet aggregation and blood fibrinolytic activity (Kendler, 1987, Preventative Medicine, 16:670-685).

The medicinal effects of garlic are generally ascribed to its oil and water soluble sulfur containing compounds (though its selenium content may also play a role). The most
20 important of these has been considered to be the amino acid alliin (representing about 70% of the total amount of sulfur in fresh garlic) which is converted through the enzyme allinase into allicin. Allicin is unstable and further metabolized into other compounds (Siems et al., 1995, Int. Symp. on Natural Antioxidants: Molecular Mechanisms and
25 Health Effects, Peking, China, pp. 188-195). These researchers stated that garlic seems to be an effective natural antioxidant and could play a role in the prevention and treatment of chronic and degenerative diseases connected with lipid peroxidation and accelerated free radical
30 generation.

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One of garlic's components, ajoene, has shown antiplatelet activity by direct interaction with the fibrinogen receptor (Apitz-Castro et al., 1986, *Biochemical and Biophysical Research Communications*, 141:145-150). It has also been described as a powerful antithrombotic agent (Block and Ahmad, 1984, *J. Chem. Soc.* 106:8295-8296). Fibrinolytic activity of garlic essential oil has been shown in patients with myocardial infarction (Bordia et al., 1977, *Atherosclerosis*, 28:155-159).

Other garlic constituents such as S-allylmercaptocysteine (ASSC) and S-methylmercapto-cysteine (MSSC) are also thought to exert bioactive effects. For example, ASSC and MSSC are known to exert significant antihepatotoxic effects (Hikino et al., 1985, *Planta Med.* 163-168).

Significant reviews of garlic's pharmacology and medical applications have been written recently (Reuter, 1995, *Phytomedicine* 2:73-91; Srivastava et al., 1995, *South African Journal of Science*, 91:68-77) including a book edited by Koch and Lawson (1996, Garlic: The Science and Therapeutic Application of *Allium sativum* L. and Related Species, 2nd ed., Williams & Wilkins, Baltimore.)

3. SUMMARY OF THE INVENTION

This invention provides a method for making a pharmaceutical grade botanical drug, for example, of garlic. The invention relates to a method for determining whether a botanical material of garlic is pharmaceutical grade. The method is the process of PharmaPrinting™. In one embodiment, the method comprises the steps of: providing a botanical material of garlic which comprises a plurality of components which have a given biological activity; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions

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wherein each of the marker fractions comprises at least one of the active components; determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the aliquot; and comparing the bioactivity fingerprint of the aliquot to a
5 bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade garlic based on the bioactivity fingerprint comparison.

This invention also provides a method comprising the
10 steps of: providing a botanical material of garlic which has a given biological activity, said botanical material comprising a plurality of components; separating a representative aliquot of the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;
15 determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to determine whether the
20 botanical material is a pharmaceutical grade garlic.

In one embodiment, one or more of the marker fractions contain one active component.

The method may also comprise the additional steps of: determining the amount of the active components in each of the marker fractions to provide a quantitative compositional
25 fingerprint of the aliquot and comparing both the quantitative compositional and bioactivity fingerprints with a quantitative compositional and bioactivity fingerprint standard to determine whether the botanical material is a pharmaceutical grade garlic. The method may also comprise the additional steps of: determining a total bioactivity of
30 the aliquot of the botanical material and comparing the total bioactivity of the aliquot with that of a total bioactivity

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of a standard which has been established for a pharmaceutical grade garlic.

The invention also provides a method for making a pharmaceutical grade garlic, the method comprising the steps of: providing a botanical material of garlic which comprises
5 a plurality of components which have a given biological activity and wherein each active component has a standardized bioactivity profile; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions wherein each of the marker fractions comprises at least one of the active components;
10 measuring the amount of each of the active component(s) present in each of the marker fractions; calculating the bioactivity of each of the marker fractions based on the amount of each active component present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the aliquot; comparing the
15 calculated bioactivity fingerprint of the aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade garlic based on the bioactivity fingerprint comparison.

20 The method of the invention is useful to make a pharmaceutical grade botanical material, e.g., garlic from an appropriate botanical material which has a given or desired biological activity. Preferably, the botanical material is an extract made from plant material such as an aqueous or organic extract such as an alcoholic extract or a
25 supercritical carbon dioxide extract or organic solvent extract which may be subject to further processing. Alternatively, the botanical material is a powdered plant material, a seed oil, an essential oil or the product of steam distillation. In one embodiment, the botanical material is a homogeneous material in a single physical
30 state, e.g. an oil or a solution. The botanical material may

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be a pure material derived solely from the botanical of interest.

In this invention the active component(s) include, but are not limited to, one or more of the following chemical classes: acetogenins, alkaloids, carbohydrates, carotenoids, 5 cinnamic acid derivatives, fatty acids, fatty acid esters, flavonoids, glycosides, isoprenoids, lipids, lipophilic compounds, macrocyclic antibiotics, nucleic acids, penicillins, peptides, phenolics, polyacetylenes, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids, sulfur containing compounds and 10 terpenoids.

The bioactivity/clinical indication for the garlic may be associated with a disease, disorder or condition of humans or other animals. Thus the methods are useful to produce pharmaceutical grade garlic for treatment and/or amelioration and/or prevention of human and/or veterinary diseases, 15 disorders or conditions. Exemplary indications include, but are not limited to, the prevention and treatment of arteriosclerosis, treatment of hypertension, hyperglycemia, digestive ailments, colds, flu, bronchitis and use as an antibacterial and antifungal.

In these methods, the aliquot may be separated into both 20 biologically active and inactive components. Furthermore, the marker fractions may comprise a class of related components.

This invention also provides a method of preparing a PharmaPrint® for a pharmaceutical grade botanical, e.g., garlic. Furthermore, this invention provides for a 25 pharmaceutical grade botanical, e.g., garlic, prepared by the methods described herein.

The present invention also provides a method for making a pharmaceutical grade garlic as described above wherein the active component is selected from the group consisting of lipids, lipophilic substances, prostaglandins, and sulfur 30 containing compounds. Further, the invention provides a method for making a pharmaceutical grade garlic as described

above wherein the active component is a sulfur containing compound which is allicin. The invention also provides a method for making a pharmaceutical grade garlic as described above wherein the active component is a sulfur containing compound which is ajoene. Additionally, the invention
5 provides a method for making a pharmaceutical grade garlic as described above wherein the active component is a sulfur containing compound which is S-allylmercaptocysteine. The invention further provides a method for making a pharmaceutical grade garlic as described above wherein the active component is a sulfur containing compound which is S-
10 methylmercaptocysteine.

In an alternative embodiment, garlic may be combined with one or more botanical materials selected from: aloe, astragalus, bilberry, black cohosh, burdock, chamomile, chaste tree, chestnut, coriolus versicolor, couchgrass, crampbark, dandelion root, dong quai, echinacea, elecampane,
15 evening primrose, eyebright, false unicorn root, feverfew, ginger, ginkgo, ginseng (Asian or Siberian varieties), goldenseal, gota kola, grape seed extract, green tea, guggulipid, hawthorn, hops, ivy, kava, licorice, milk thistle, mistletoes (American, Asian and European varieties), motherwort, oats, osha, passion flower, pumpkin, pygeum, red
20 clover, rosemary, sarsaparilla, saw palmetto, skullcap, St. John's wort, stinging nettle, valerian, wild indigo, wild yam, and yerba mansa. The methods of the present invention for making pharmaceutical drugs encompass methods for PharmaPrinting™ garlic plus one or more of the botanicals
25 listed above as well as pharmaceutical grade drugs containing garlic and one or more of the botanicals listed above. In one mode of this embodiment, garlic may be combined with hawthorn and/or motherwort.

3.1. DEFINITIONS

30 The term "pharmaceutical grade" when used in this specification means that certain specified biologically

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active and/or inactive components in a botanical drug must be within certain specified absolute and/or relative concentration range and/or that the components must exhibit certain activity levels as measured by a disease-, disorder- or condition-specific bioactivity assay. The disease,
5 disorder or condition may afflict a human or an animal.

As will be understood by those skilled in the art, the term "pharmaceutical grade" is not meant to imply that the botanical drug is applicable only to products which are regulated for example those provided under prescription, i.e., "Rx" products or over the counter, i.e., "OTC". The
10 term is equally applicable to products provided Rx, OTC or as a dietary supplement, i.e., "DSHEA".

As used herein "components" means discrete compounds (i.e. chemicals) which either are present naturally in a botanical drug or have been added to the botanical drug so as
15 to prepare a pharmaceutical grade botanical drug having components within a defined bioactivity range(s) and/or compositional range(s).

As used herein "active components(s)" means one or more component(s) for which the summation of the individual component(s) activity in a disease-specific bioassay accounts
20 for a substantial portion of the observed biological activity of the botanical material. Preferably, the summation of the active components' activities accounts for the majority or greater than 50% of the observed biological activity.

As used herein "fractions" typically means a group of components or class of structurally similar components having
25 defined parameters such as solubility, molecular weight range, polarity range, adsorption coefficients, binding characteristics, chemical reactivity or selective solubility. Most frequently fractions will be the product of selective solvent solubility and partition techniques (i.e. liquid-liquid extraction) including pH dependent separations,
30 chromatographic separation techniques, i.e., flash chromatography, preparative high performance liquid

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chromatography (HPLC), preparative gas chromatography, partition chromatography, preparative thin layer chromatography, affinity chromatography, size exclusion chromatography, liquid-liquid chromatography e.g., counter-current chromatography or centripetal or centrifugal
5 chromatography.

The present invention may be understood more fully by reference to the detailed description of the invention and examples of specific embodiments and the appended figures.

10 4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a procedure in accordance with the present invention which is used to establish standard chemical and/or bioactivity fingerprints against which subsequent processed botanical materials are compared during production of pharmaceutical grade drugs.

15 FIG. 2 is a schematic representation of a procedure in accordance with the present invention which is used to process botanical materials into pharmaceutical grade drugs.

FIG. 3 is a schematic representation of a procedure for
20 isolating different classes of biologically active components.

5. DETAILED DESCRIPTION OF THE INVENTION

25 5.1. METHODS OF PHARMAPRINTING™

The present invention provides a method for producing botanical drugs which may be classified as being of pharmaceutical grade. The method is designated PharmaPrinting™. The pharmaceutical grade botanical drugs made by the method of the present invention are particularly
30 well-suited for use in clinical studies and more importantly for use in treatment of patients. The method insures that

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the drug being used for a particular protocol will be of consistent quality and consistently suitable for use as human and veterinary prophylactic or therapeutic agents.

The present invention provides the ability to closely control the quality, dosage and clinical effectiveness of botanical extracts and other botanical materials, e.g., botanical material(s) of garlic. One aspect of the present invention involves the establishment of the chemical and/or bioactivity fingerprint standards for various botanical materials. Once established, the fingerprint standards are used in drug production procedures to insure that the botanical materials meet pharmaceutical grade requirements. Specific quantitative and biological fingerprints are presented which have been established for a number of botanical materials as a further aspect of the invention. These fingerprints are useful for determining if a particular botanical material meets levels of pharmacological activity and composition requirements for a particular treatment regimen. Such a determination is important to insure that clinical studies and patient treatment with the botanical materials are based on consistent and verifiable extract composition parameters.

This invention is useful in providing botanical materials which are sufficiently characterized and whose compositions are consistent between batches, so that they can be precisely dosed and used effectively in clinical settings. The methods described herein provide an assurance that the results of a clinical trial will be reproducible.

Initially, a sample of the botanical material of interest is obtained. Many botanicals are commercially available as the raw material or as a processed extract. Often it is a botanical extract or other composition which is intended for use as a drug. The processed material may include a plurality of active components which exhibit a given biological activity and plurality of inactive components which do not directly exhibit the biological

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activity of interest. In one embodiment, an aliquot is removed from the botanical material and subjected to a quality assurance or standardization procedure. Preferably, the aliquot is a representative aliquot of a homogeneous botanical material. The procedure involves separating the
5 aliquot of botanical material into a plurality of marker fractions wherein each of the marker fractions includes at least one of the active components or in some cases one of the inactive components. The amount of active component or inactive component in each of the marker fractions is determined in order to provide a quantitative fingerprint of
10 the aliquot. The degree of biological activity for each of the marker fractions is also determined to provide a biological activity fingerprint for the aliquot. The chemical and/or biological activity fingerprints of the aliquot are then compared to corresponding fingerprints which have been established for a pharmaceutical grade drug. If
15 the fingerprints of the botanical match the standard fingerprints, then the botanical is identified as a pharmaceutical grade botanical drug. If not, then the botanical may be modified so as to provide a match with the standard fingerprints or may be rejected.

20 **5.1.1. METHODS OF DEVELOPING A PHARMAPRINT®**

The method of developing a PharmaPrint® for a botanical when a range of putative active components is known begins with a literature review. It involves reviewing the chemical literature, the biological literature, the published
25 bioassays and clinical data for the botanical. Particularly useful sources of information are the NAPRALERT computer database managed by Dr. Norman Farnsworth in the Program for Collaborative Research in the Pharmaceutical Sciences, University of Illinois, Chicago; Leung and Foster, Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics, 2nd Ed. John Wiley & Sons: New York, NY,
30 1996; Herbal Drugs and Phytopharmaceuticals, ed. N.G. Bisset,

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CRC Press: Boca Raton, FL, 1994; Duke, Handbook of Biologically Active Phytochemicals and Their Activities, CRC Press: Boca Raton, FL, 1992; Tyler and Foster "Herbs and Phytomedicinal Products" in Handbook of Nonprescription Drugs Berardi et al. eds., United Book Press, Inc.: Washington, DC, 5 1996. For a given indication, the literature must be studied to confirm that the putative active components are actually associated with that disease state. In addition, if there are any bioassays known for the putative active components and known for the indication, the bioassays must be consistent with both the indication and the putative active 10 components. The appropriate bioassay(s) is tied to a clinically relevant endpoint(s). The bioassay(s) should be quantitative over a wide concentration range. Typically, an IC_{50} curve (Inhibitory Concentration 50%), EC_{50} (Effective Concentration 50%), or an appropriate K_i or K_o (dissociation constant of the enzyme and its inhibitor) curve is prepared. 15 A thorough chemical and biological analysis of both putative active components and chromatographic fractions of the botanical is then performed. The results are analyzed to prepare a quantitative analysis of the biological activity for each of the chemical components in the sample. Then, the bioactivity of the sample as a whole is compared to the 20 bioactivity of the individual components. At this point the individual chemical components can be correlated with a clinically relevant endpoint. Similar methodologies may be applied to bioassays measuring stimulatory or inhibitory effects.

Based on activity of the components individually and 25 knowing the total activity, the components should, when combined, account for a substantial portion of the biological activity. Generally, the combined activity will account for at least 25% of the total activity.

Preferably, the summation of the individual active components' activities account for the majority or greater 30 than 50% of the observed biological activity. More preferably, the isolated individual components are

responsible for more than 70% of the activity. More preferable still, the isolated individual components are responsible for greater than 80% of the biological activity.

Another consideration will be to select as few active components as possible to be part of the PharmaPrint™. Fewer
5 active components are important for practical considerations in raw material acceptance and manufacturing. In this invention, a correlation is established between the relevant chemical components and the bioactivity. Once a satisfactory correlation is established, it may not be necessary to perform the biological fingerprints on each sample. Rather,
10 a chemical analysis of the appropriate components and/or marker fractions of each sample of the botanical of interest will suffice to account for most of the biological activity and establish that a given botanical sample is pharmaceutical grade.

In one embodiment, the present invention may involve one
15 of the following procedures. One procedure, as schematically outlined in FIG. 1, involves establishing the compositional and bioactivity fingerprint standards for a given pharmaceutical grade botanical drug. Once the fingerprint standards are established, then the actual processing of the botanical into a pharmaceutical grade drug can be carried out
20 as schematically outlined in FIG. 2.

The initial step in establishing the chemical and/or bioactivity fingerprint for a given botanical involves separating the extract or powder into one or more groups as represented by step 1 in FIG. 1. These groups are separated out and identified based on their potential as markers (which
25 may or may not comprise active components) for the fingerprint which is to be established for the processed botanical material. The putative components or groups of putative components which are chosen and identified as potential markers will vary widely depending upon the botanical being processed and the pharmaceutical use. There
30 should be at least two putative markers selected for each botanical. The number of potential markers may be more than

five and can be as high 15 to 20 or more for complex botanical extracts or powders. The potential markers are identified and selected, for the most part, based on their potential biological activity or contribution to biological activity for a given pharmaceutical application. For a
5 different indication the same botanical may be used for preparing an extract with a different extraction procedure in order to optimize specific bioactive constituents. Markers which have no apparent biological activity by themselves may be separated out and may be included as markers for use in the fingerprint. These "proxy" markers may be desirable as
10 an internal standard where the markers' presence is indicative of other active components necessary to provide a substantial portion of the overall observed biological activity for the botanical drug. They also help to assure proper botanical identity of the drug (i.e. chemotaxonomy).

The initial separation of the botanical into various
15 groups of putative markers is accomplished by conventional separation techniques ranging from simple extraction and partition, to complex affinity chromatographic techniques, including gel filtration chromatography, flash silica gel chromatography and reverse phase chromatography. Once the putative markers have been identified for a given botanical,
20 then the bioactivity of each of the markers is determined as depicted by step 2 in FIG. 1. The particular bioassay used to determine bioactivity of the botanical is chosen based upon the intended use for the botanical. The bioassay preferably will provide a reflection of the putative markers' bioactivity with respect to the condition or indication which
25 is to be treated with the botanical.

The bioassay results obtained in step 2 are used to identify the components having the desired bioactivity (step 3) and those which are less active or essentially inactive (step 4). Each of the groups identified in steps 3 and 4 is then analyzed quantitatively to determine the amount of each
30 identified component present in each group. The results of the bioassays and quantitative compositional assays are then

used to prepare a bioassay fingerprint and/or a chemical fingerprint for the botanical as depicted by step 5 in FIG.

1. As part of establishing the fingerprints for the botanical, acceptable ranges of bioactivity and/or chemical composition are determined. This is done primarily based
5 upon establishing acceptable ranges of bioactivity and quantitative amounts for each marker which provide for the desired pharmacological activity of the processed material as a whole.

In addition, various combinations of active and inactive marker fractions may be evaluated to establish potential
10 increases in desired bioactivity resulting from combinations of the active and inactive components.

The bioassay and quantitative fingerprints which are established in step 5 provide an accurate identification of the botanical which can be used in establishing the dosage regimens and treatment schedules which are necessary for
15 clinical use. The dosage regimens and treatment schedules are established using conventional clinical methods which are commonly employed when investigating any new drug. The processed material which is used to determine the dosage and treatment schedules must be matched with and meet the requirements of the fingerprints established in step 5. This
20 method insures that the dosage and treatment schedules are effective and reproducible since the processed materials used in the dosage and scheduling studies all have the same fingerprints in accordance with the present invention.

The bioassay and quantitative fingerprints which are determined by the general procedure as set forth in FIG. 1
25 are used as part of the manufacturing procedure for producing pharmaceutical grade botanical drugs. The fingerprints are used as part of a quality assurance or standardization procedure to insure that a given botanical contains the appropriate compounds and is processed correctly to provide a botanical drug which will perform the same clinically as the
30 material which has been standardized and tested in accordance with the procedure set forth in FIG. 1.

An exemplary procedure for producing pharmaceutical grade botanicals in accordance with the present invention is shown schematically in FIG. 2. The botanical material of interest 21 is first processed by extraction, powdering or other manufacturing process to form a processed botanical material 22. A sample of the processed material 22 is then analyzed to establish whether or not it matches the fingerprint requirements established during the standardization procedure of FIG. 1. This quality assurance or standardization procedure is depicted at step 23 in FIG. 2. If the processed material meets the previously established fingerprint requirements for the particular material, then it is approved as being of pharmaceutical grade as represented by step 24. If the material is close, but does not quite match the standard fingerprint, then it is modified as required to match the fingerprint standards (step 25). The modification of the processed material to meet fingerprint standards may be done by a variety of ways. The methods of further processing botanicals may including additional extraction of the botanical, selective extraction, selective processing, recombination of batches (e.g. mixing high and low dose batches to prepare the pharmaceutical grade material) or the addition of various compounds, as required. If the botanical is substantially outside the fingerprint ranges for both bioactivity markers and quantitative markers, then the batch is rejected (step 26).

In one embodiment, the quality assurance standardization step 23 used to determine if a given botanical is pharmaceutical grade involves obtaining a uniform sample, preferably a homogeneous sample, or aliquot of the botanical which is to be tested. The sample should include the active components which contribute to the observed biological activity of the material and produce the bioactivity and/or chemical fingerprint of the previously determined standard. The sample will also include one or more inactive components. Inactive components are those which may not have a direct

measurable biological activity. Inactive components include the following categories: components with activity so low that they do not account for a substantial portion of the activity; components whose presence indicates the presence of other bioactive components and can act as proxy markers for these components; inactive components that are chemically or biologically inactive in the relevant assays. The sample is preferably only a small aliquot of the botanical material being tested. Accordingly, it is important that a uniform sample, preferably a homogeneous sample, be obtained which is representative of the entire batch of material.

10 A more detailed schematic is shown in FIG. 3 showing the initial separation of the different components present in an aqueous extract of a botanical. Sequential extraction and precipitation are used to isolate the active components in either the aqueous or the organic phase. The scheme in FIG. 3 is particularly well suited for separating the classes of 15 water-soluble active components from a botanical such as mistletoe.

An exemplary general method for separating plants into major classes of chemical components is set forth schematically in FIG. 3. Primarily fresh plants (including leaves, roots, flowers, berries and stems) should be used, 20 although dried materials may also be utilized. Specific plant parts, such as the leaves, flowers, stems or root may be used if desired.

In this method the specific part or whole plant may be frozen at liquid nitrogen temperature. This facilitates grinding and also preserves the integrity and potency of the 25 active components.

The pulverized powder is extracted with distilled water repeatedly. If desired, the extraction may be carried out with hot water, alcohol, other organic solvents, aqueous alcohol, dilute acetic acid or any combination thereof. The actual temperature chosen is preferably close to or at the 30 boiling temperature of water. It is preferred that the overall bioactivity of the extract be initially determined.

The combined extracts are subjected to a specific bioassay, e.g., a test for inhibiting the growth of bacteria in Petri dishes if the drug is to be used as an antibacterial. Alternatively, tests against cell cultures of cancer cells are conducted preferably if the drug is intended for use as an anticancer agent. From these data, bioactivity units contained in an extract per ml are calculated (bioactivity units are defined as the dilution number of this extract needed to inhibit 50% growth of bacterium or cancer cell in test system). Similarly bioactivity units for a stimulatory effect, e.g., immunostimulation can be calculated.

For establishing a pharmaceutical fingerprint (PharmaPrint®) in accordance with the present invention, the plant is extracted according to the procedure as set forth in FIG. 3 to separate it into major components (e.g. saponins, terpenoids, lipids, alkaloids, nucleic acids, proteins and carbohydrates). Each separated group of components is tested for bioactivity as needed. This may point to activity (e.g. in protein and alkaloid fractions as in *Viscum album*). The active class or classes of compounds are further separated into individual components by affinity chromatography, high performance liquid chromatography, gas chromatography or other chromatography. The components with major contribution towards biological activity are quantified on the basis of weight and specific bioactivity units. These components provide the fingerprint to establish the pharmaceutical requirements for the original herbal extract. The bioactivity units per ml of the pharmaceutical grade extract provide a way to establish exact dosage for clinical studies.

Once the sample is separated into individual marker fractions, and at least one having at least one active component, each fraction is analyzed to determine the amount of active component therein and provide a quantitative fingerprint of the sample. The quantitation of each fraction can be achieved using any of the known quantitative analysis methods. Exemplary quantitation methods include gravimetric

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analysis, spectral analysis or the use of quantitative detectors, such as those used in gas chromatography or high performance liquid chromatography and other separation systems. Other suitable quantitative analytical methods include analysis by enzymatic, radiometric, colorimetric, 5 elemental analysis spectrophotometric, fluorescent or phosphorescent methods and antibody assays such as enzyme linked immunosorbant assay (ELISA) or radioimmunoassay (RIA).

In one embodiment, the results of the quantitative analysis of each fraction are used to prepare a quantitative fingerprint of the sample. The fingerprint is composed of 10 the quantity of component in each of the marker fractions and the identity of the component. This quantitative fingerprint is then compared to the known standard fingerprint which has been established (FIG. 1) in order for the material to be considered as pharmaceutical grade. If the quantitative fingerprint of the sample falls within the range of 15 quantities set forth for the pharmaceutical grade fingerprint, then the material may be identified as being of pharmaceutical grade.

As a further part of the quality assurance assay, the individual marker fractions may be subjected to biological assays. The biological assays which are used to test the 20 various fractions are the same as those used for the standard fingerprint and will also depend upon the particular clinical use intended for the material.

The bioactivity fingerprint generated for the material is compared to the standard bioactivity fingerprint which has been established in order for the material to be considered 25 as pharmaceutical grade. If the bioactivity fingerprint of the sample falls within the range of bioactivities set forth for the pharmaceutical grade fingerprint, then the material is identified as, and approved as, being of pharmaceutical grade.

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5.1.2. ALTERNATIVE METHODS OF DEVELOPING A PHARMAPRINT®

The method of developing a PharmaPrint® for a botanical when the putative active components are not known also begins with a literature review. It involves reviewing any chemical literature, biological literature, published bioassays or
5 clinical data available for the botanical, or related botanicals, or for botanicals with related activities. Based on the disease state, a series of relevant bioassays is chosen. The activity of the total sample or extract is analyzed using bioassays. Those bioassays that show activity are then used to analyze fractions of the botanical for which
10 the putative active components are not yet known. The fractionation is based on the usual methods, e.g., separation by dielectric constant, biological affinity, polarity, size, solubility or absorptive power. The fractions are then analyzed to determine which fraction is responsible for the activity. Assuming activity is found, each active fraction
15 is refractionated to isolate the individual putative active components, i.e., pure chemical compounds. Based on knowing the individual chemical compounds and knowing their quantitative biological activity, a quantitative potency curve may be drawn and the 50% inhibitory concentration (IC_{50})
20 for each individual chemical component may be determined. If the putative active components are agonists, then other parameters (binding, activation, response) may be needed. In the general case, the bioassay will consist of appropriate tests of the stimulatory or inhibitory effects of the constituents, fractions or entire extract, followed by an
25 appropriate quantitative evaluation of those effects. For the most likely (or typical) assays in which a standard (or radiolabelled) agonist or antagonist causes a measurable effect, inhibition and/or stimulation by the subject material may be assessed and expressed typically via the determination of an IC_{50} , EC_{50} , etc. value, or other suitable measure (e.g.,
30 K_i , K_d , K_m , etc). The activities of individual putative active components are then totaled and that summation is

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compared to the activity in the unfractionated botanical sample. If these components account for a substantial portion of the activity, then one has an initial fingerprint of "active components" for the botanical where the active components were not known.

5

5.1.3 ADDITIONAL VARIATIONS ON THE METHODS OF DEVELOPING A PHARMAPRINT®

The general methods outlined above for PharmaPrinting™ a botanical whose putative active components are not known have several variations should complications arise in the course
10 of the analysis. One variation occurs when the summation of individual components do not account for a substantial portion of the biological activity of the botanical. At this point there are several likely reasons for the reduced activity of the individual components, one, decomposition or degradation of active components or, two, a synergistic
15 effect. In another possible scenario there may be no significant or greatly lessened activity seen from any of the fractions, but the whole botanical or extract shows activity in the bioassay. Nonspecific matrix effects may also lessen the total extract activity, when compared to standards.

To determine if the active components are decomposing in
20 the course of the assay is relatively simple. One merely recombines all of the fractions and compares the activity of the recombined fractions with the activity of the crude material. If substantial activity has been lost, then the problem is probably decomposition. To determine which active components may be decomposing, the chromatographic analysis
25 of the crude botanical is compared with that of the recombined fractions. Peaks that are missing or are reduced in size indicate that components may be decomposing. To overcome decomposition many methods exist. Typically, milder extraction/fractionation methods such as liquid-liquid chromatography (counter-current chromatography) or
30 supercritical carbon dioxide extraction or chromatography may be used.

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Another explanation for the activity of the individual fractions not accounting for a substantial portion of the expected total activity is a synergistic effect between one or more active components with each other, or inactive components. To determine that a synergistic effect is taking
5 place, pair-wise recombined fractions need to be analyzed. If the combined fractions show more activity than the individual fractions, two or more individual components in the fractions may be acting synergistically. For example, one may have three fractions, each alone responsible for 10% of the bioactivity (i.e., their uncombined additive
10 bioactivity is 30%) but combined responsible for 100% of the activity. In that case the fractions are acting synergistically. By repeated pair-wise recombination of fractions or looking at larger fractions, any synergistic activity will be discovered. Once two fractions show synergy, they are then refractionated as above, and pairs of
15 individual fractions or pairs of isolated components are studied to find the individual components that act synergistically. Three way comparisons of individual components or fractions may also be studied.

What if the fractions have no activity in the bioassay in which the botanical shows activity? Here, the
20 explanations include decomposition, synergy, or many active components such that no individual fraction shows activity. The first step would be to fractionate each initial fraction and see if active components appear in the bioassay. If that does not succeed, the fractions should be recombined and assayed to determine if decomposition of the actives is
25 taking place. If decomposition is taking place, the appropriate measures as described above should be taken. If there is no decomposition, then alternative methods of fractionation should be tried. Eventually, large enough or appropriately sized or selected fractions will show activity. If synergy is a suspected problem, then proceed as in the
30 synergy section described above.

5.2. METHODS OF PROCESSING AND EXTRACTING BOTANICAL MATERIALS

The botanical material, for example garlic, may be processed to form an aqueous or organic extract of the whole
5 plant or a selected part of the plant. The botanical material can also be processed in whole or part to form a powder. Many of the botanicals of interest are commercially available as powders, aqueous extracts, organic extracts or oils. In one embodiment, extracts of the plant material are preferred because they are easier to dissolve in liquid
10 pharmaceutical carriers. However, powdered plant materials are well-suited for many applications where the drug is administered in solid form, e.g., tablets or capsules. Such methods are well known to those of skill in the art. Furthermore, many of the plant materials and/or extracts are available commercially. As examples of the processing and
15 extracting of botanicals the following examples are provided. Additional examples are provided in the detailed description.

For a typical root, it may be sliced, frozen or pulverized. If powdered it is then shaken with an appropriate solvent and filtered (Tanabe et al., 1991, *Shoyakugaku Zasshi*, 45(4):316-320). Alternatively, the
20 following methods are used: the root is homogenized, acetone extracted and filtered; the botanical may be steam distilled to obtain essential oils and the distillate dissolved in acetone-water or appropriate solvent; or the cut rhizomes are frozen and/or freeze-dried and the resulting powder acetone-water extracted (Tanabe et al., 1991, *Shoyakugaku Zasshi*
25 45(4):321-326). Another method of processing botanicals is aqueous extraction with 100°C water (Yamahara et al., 1985, *J. Ethnopharmacology* 13:217-225). The initial solvent extract from the methods above may be further extracted using liquid/liquid extraction with an appropriate solvent. The
30 botanical may be extracted in two steps using polar and non-polar solvents respectively. The solvents are then

evaporated and the fractions combined (Nagabhusan et al., 1987, *Cancer Let.* 36:221-233). Botanicals may also be processed as a paste or powder which may be cooked (Zhang et al., 1994, *J. of Food Science* 59(6):1338-1343).

5 A variety of solvents may be used to extract the dried botanicals, for example acetone, acetonitrile, dichloromethane, ethyl acetate, ethanol, hexane, isopropanol, methanol, other alcohols, and supercritical carbon dioxide (Sipro et al., 1990, *Int. J. of Food Science and Technology* 25:566-575 and references therein).

10 For other botanicals such as Saw Palmetto, the medicinal products are the seed oil or dried berries. In a typical preparation, a hexane or supercritical carbon dioxide extract is prepared. Many Saw Palmetto preparations are commercially available, for example Permixon™ or Talso™. For an example of supercritical carbon dioxide extraction of a botanical,
15 see Indena, European Patent No. 0 250 953 B1. Alternatively, the botanical may be crushed and extracted with an appropriate solvent (90%) in a soxhlet (Elghamry et al., 1969, *Experientia* 25(8):828-829). The botanical may also be ethanol extracted (Weisser et al., 1996, *The Prostate* 28:300-306).

20 The dried material may be prepared in a variety of ways including freeze-drying, drying via microwave, cooling with liquid nitrogen and pulverizing; drying at 70°C under vacuum for a duration of 10 hours; or air-drying in the shade, or with forced heated air (List and Schmidt, Hagers Handbuch der Pharmazeutischen Praxis, Springer-Verlag: New York, 1993,
25 1973-79; Araya et al., 1981, *Journal of Comparative Pathology*, 135-141). Teas, dilute aqueous extracts, also known as infusions, may be made in 60-100°C water (Nosel and Schilcher, 1990). Decoctions may also be utilized. Extraction is more efficient when the particle size is less
30 than .25 mm (List and Schmidt, Phytopharmaceutical Technology, CRC Press: Boca Raton, FL, 1989).

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Various guidelines are available for preparing oil extracts of botanicals. The botanical may be digested (macerated) in oil at 45°C for 10 days, while others recommend 70°C for 12-24 hours (Hobbs, 1989, *HerbalGram* 18/19:24-33; Smith et al., Quality Validation Laboratory - Herb Pharm: Williams, OR, 1996). In St. John's Wort for example, exposing the preparation to sunlight during the extraction process has been reported to result in a four-fold increase in flavonoid content calculated as quercetin (Maisenbacher and Kovar, 1992). Additionally, for St. John's Wort, two-fold increases of hypericin have been reported in oil preparations in which the material has been further extracted with alcohol, and mixed with the oil (Georgiev et al., 1983, *Nauchni Tr.-Vissh Inst. Plovid.* 30:175-183).

Alternatively an alcohol-water preparation may be prepared of the botanical (Dyukova, 1985, *Farmitsiya* 34:71-72; Georgiev et al., 1985, *Nauchni Tr.-Vissh Inst. Plovid.* 32:257-263; Wagner and Bladt, 1994, Kowalewski et al., 1981, *Herba Pol.* 27:295-302). According to Hagers Handbuch a tincture of a botanical, such as St. John's Wort, may be prepared by using drug or freezing ethanol soaked botanical materials, and filtering and preserving in dark bottles (List and Hörhammer, 1993).

Some botanicals, such as St. John's Wort, are both temperature and light sensitive. For this type of botanical the material should be dry packed with a refrigerant or shipped under refrigeration and protected from light and air. In St. John's Wort, hypericin content has been shown to drop significantly in powdered extract, tablet and juice preparations when stored at temperatures of 60°C-140°C for more than six weeks. Dry extracts stored at 20°C were found to remain stable for at least one year (Adamski et al., 1971, *Farm. Pol.* 27:237-241; Benigni et al. Hypericum. Plante Medicinali: Chimica, Farmacologia e Terapia, Milano: Invernizzi & Della Beffa; 1971). Other St. John's Wort constituents,

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hyperforin and adhyperforin found in oil preparations are highly unstable, especially when exposed to light, and can degrade in as little as 14 days (Meisenbacher et al., 1992, *Planta Med.*, 351-354). Stability (in absence of air) was increased to six months in a preparation extracted with ethanol. Similarly, up to four xanthones and several flavonoids including quercetin and I3', II8-biapigenin have been detected suggesting these may be among the active constituents in external preparations (Bystrov et al., 1975, *Tetrahedron Letters* 32:2791-2794).

10

5.2.1. EXTRACTS OF PLANT MATERIALS AND POWDERED PLANT MATERIALS

Garlic is typically provided as a botanical material which is fresh, in an oil form, or dried.

One common form of liquid extract of botanical material is a "tea". A tea may be prepared through processes of infusion or decoction. Teas are generally an effective means to extract water soluble components from dried or fresh botanicals.

Another common form of liquid botanical extract is a tincture. A botanical tincture is typically an alcoholic or hydroalcoholic solution prepared from a fresh or dried botanical. It is usually prepared through a process of percolation or maceration.

Tinctures of potent botanicals, and homeopathic mother tinctures, may represent 10 g of botanical (dry weight) in 100 ml of tincture. Common botanicals have 20 g of botanical represented in 100 ml of tincture. The respective ratios of dried botanical to solvent for these preparations are 1:10 and 1:5, respectively. While these concentrations have been officially recognized by the U.S. National Formulary it has become common for tinctures to be prepared in 1:4, and other concentrations.

As compared to crude botanical extracts, tinctures may have a reduced microbial load and longer shelf life. This is

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largely due to the presence of alcohol at 20% or greater concentrations in the extract. Occasionally liquid extracts are made with glycerin and water as the solvent. These glycerites usually need to have at least 50% glycerin present to inhibit microbial contamination. Glycerites may also be
5 prepared from tinctures by evaporating off alcohol and "back adding" glycerin in its place.

Another type of liquid extract is a "fluid extract". Fluid extracts are liquid preparations of botanicals that represent the medicinal properties of 1 g of dried botanical in 1 ml of extract. Official versions are made by the
10 percolation process according to official monographs which determine the solvent to be used.

Liquid extracts that are concentrated, usually through evaporation of the solvent, may form extracts that are oily, semi-solid, or solid in nature.

Dry powdered extracts may be prepared by the absorption
15 of liquid extracts, oils, or semi-oils onto suitable carriers before solvent removal. Alternatively, dry powdered extracts may be prepared by direct removal of solvents from a liquid extract to provide a solid extract which can be powdered.

20

5.3 SEPARATION OF FRACTIONS

Once the botanical extract has been prepared and/or alternatively purchased as a commercially available extract, a portion needs to be subjected to fractional analysis. If the fingerprint has already been established, the sample or aliquot is separated into the same plurality of marker
25 fractions which are present in the standard fingerprint. Each of the marker fractions will include one or more of the active or inactive components. The marker fractions are established on an individual basis for each botanical material being tested. For some materials only a few marker fractions are required. For other more complex materials,
30 there may be numerous marker fractions. For example in

mistletoe, *Viscum album* L. protein extract, the preferred protein marker fractions are those fractions which are separated based on the sugar binding affinity of the fraction. However, different parameters for identifying and separating the materials into the marker fractions may be
5 established based upon the types of components present in the botanical material. Separation of the sample into the marker fractions may be accomplished by any of the conventional separation techniques including liquid chromatography and extraction procedures. The same procedures which were used to establish the standard fingerprints should be used. Since
10 the various fractions may be tested for biological activity, it is preferred that non-destructive separation techniques be utilized. Liquid column chromatography is a useful separation technique with affinity chromatography based on the specific binding ability of the compounds (e.g.,
15 carbohydrates and target enzymes) being particularly used.

After the fractionation, the solvent is removed and the material is dissolved in an appropriate medium for the bioassays. Examples of appropriate media include DMSO, ethanol, various detergents, water and an appropriate buffer. The choice of solvent will depend on the chemical nature of
20 the component being analyzed and the compatibility with the assay system.

5.4 ESTABLISHMENT OF APPROPRIATE BIOASSAYS

Exemplary biological assays may include any cell proliferation assays, such as the measurement of L 1210 cell
25 inhibition, immune activity or inhibition of critical enzyme which relates to specific diseases. Examples of other transformed cell lines which can be used for bioassays include HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma, hepatoma cell line, primary or established cultures of human/animal cell lines which carry specific cell
30 receptors or enzymes.

The results of the biological assays are used to prepare a bioactivity fingerprinting of the material. The fingerprint can be as simple as an assay of two selected marker fractions. Conversely, the fingerprint can include numerous different bioassays conducted on numerous different
5 fractions. The same assay may be conducted on different marker fractions. Also, different assays may be conducted on the same marker fraction. The combination of bioassays will depend upon the complexity of the given botanical material and its intended clinical use. The bioassays will be the same as those conducted in establishing bioactivity
10 fingerprint of the standard material.

5.4.1. ENZYMATIC AND RECEPTOR BASED ASSAYS

Enzymatic and receptor based assays are preferable in the practice of this invention. Assays are chosen either based on accepted enzymatic assays for a clinical disorder or
15 they are chosen from relevant assays for a given clinical disorder. It is important to choose appropriate bioassay that may be validated. Ideally, a bioassay should be rugged, that is reproducible over time and show a quantitative dose response over a wide concentration range. An issue faced with a botanical for which the active components are not
20 known is the choice of a relevant bioassay. Here, the human therapeutic use will serve as a guide to pick assays known in the art based on possible mechanisms of action. The mechanism of action should be consistent with a clinically relevant endpoint. There are a wide array of clinically relevant assays based on enzymatic activity, receptor binding
25 activity, cell culture activity, activity against tissues and whole animal in vivo activity.

This section will address enzymatic and receptor binding assays. There are many books on enzymatic or receptor assays, for example, Methods in Enzymology by Academic Press or Boyers, The Enzymes. Bioactive Natural Products,
30 Detection, Isolation, and Structural Determination, S. M.

Colegate and R. J. Molyneux, CRC Press (1993), also discusses specific bioassays. Methods in Cellular Immunology, R. Rafael Fernandez-Botran and V. Vetvicka, CRC Press (1995) describes assays from immune cell activation and cytokine receptor assays. "Screening Microbial Metabolites for New
5 Drugs-Theoretical and Practical Considerations" describes additional methods of finding pharmaceutically relevant bioassays (Yarbrough et al. (1993) *J. Antibiotics* 46(4):536-544). There are also many commercial contract research vendors, including Panlabs, Paracelsian and NovaScreen.

For example, for a botanical useful for treating
10 neurological disorders, the array of bioassays might include adrenergic receptors, cholinergic receptors, dopamine receptors, GABA receptors, glutamate receptors, monoamine oxidase, nitric oxide synthetase, opiate receptors, or serotonin receptors. For cardiovascular disorders the array of assays may include adenosine A₁ agonism and antagonism;
15 adrenergic α_1 , α_2 , β_1 agonism and antagonism; angiotensin I inhibition; platelet aggregation; calcium channel blockade; ileum contractile response; cardiac arrhythmia; cardiac inotropy; blood pressure; heart rate; chronotropy; contractility; hypoxia, hypobaric; hypoxia, KCN; portal vein, potassium depolarized; portal vein, spontaneously activated;
20 or thromboxane A₂, platelet aggregation. For metabolic disorders the following bioassays may be used: cholesterol, serum HDL, serum total; serum HDL/cholesterol ratio; HDL/LDL ratios; glucose, serum - glucose loaded; or renal function, kaluresis, saluresis, and urine volume change. For
25 allergy/inflammation disorders the following bioassays may be used: allergy, Arthurs reaction, passive cutaneous anaphylaxis; bradykinin B₂; contractility, tracheal; histamine H₁ antagonism; inflammation, carrageenan affects on macrophage migration; leukotriene D₄ antagonism; neurokinin NK₁ antagonism; or platelet activating factor, platelet
30 aggregation or induction of biosynthesis of important inflammatory mediators (e.g. interleukins IL-1, IL-6, tumor

necrosis factor or arachidonic acid). For gastrointestinal disorders the following bioassays may be used:

cholecystokinin CCK_A antagonism; cholinergic antagonism, peripheral; gastric acidity, pentagastrin; gastric ulcers, ethanol; ileum electrical stimulation modulation; ileum

5 electrical stimulation spasm or serotonin 5-HT₃ antagonism.

For antimicrobial, antifungal, or antitrichomonal disorders the following are used: *Candida albicans*; *Escherichia coli*;

Klebsiella pneumoniae; *Mycobacterium ranae*; *Proteus vulgaris*;

Pseudomonas aeruginosa; *Staphylococcus aureus*, methicillin

10 resistant; *Trichomonas foetus*; or *Trichophyton*

mentagrophytes. For other indications, one of skill in the art would be able to choose a relevant list of bioassays.

Specific examples of assays based on enzymes or receptors include the following: acetyl cholinesterase; aldol-reductase; angiotensin converting enzyme (ACE);

15 adrenergic α , β , rat androgen receptor; CNS receptors;

cyclooxygenase 1 or 2 (Cox 1, Cox 2); DNA repair enzymes;

dopamine receptors; endocrine bioassays, estrogen receptors;

fibrinogenase; GABA A or GABA B; β -glucuronidase;

lipxygenases, e.g., 5-lipxygenase; monoamine oxidases (MAO_A,

MAO_B); phospholipase A₂, platelet activating factor (PAF);

20 potassium channel assays; prostacyclin cyclin; prostaglandin synthetase; serotonin assays, e.g., 5-HT activity or other

serotonin receptor subtypes; serotonin re-uptake activity;

steroid/thyroid superfamily receptors; thromboxane synthesis activity. Specific enzymatic assays are available from a

25 variety of sources including Panlabs™ Inc (Bothell, WA) and NovaScreen™ (Baltimore, MD). Additional assays include:

ATPase inhibition, benzopyrene hydroxylase inhibition, HMG-CoA reductase inhibition, phosphodiesterase inhibition,

protease inhibition, protein biosynthesis inhibition,

tyrosine hydroxylase and kinase inhibition, testosterone-5 α -

30 reductase and cytokine receptor assays.

5.4.2 CELL CULTURE AND OTHER ASSAYS

In addition to the enzymatic and receptor assays, there are also other biological assays. Preferably, these assays are performed in cell culture but may be performed on the whole organism. Cell culture assays include activity in
5 cultured hepatocytes and hepatomas (for effect on cholesterol levels, LDL-cholesterol receptor levels and ratio of LDL/HDL cholesterol); anti-cancer activity against L 1210, HeLa or MCF-7 cells; modulating receptor levels in PC12 human neuroblastoma cells; modulation of primary cell culture
10 activity of luteinizing hormone (LH), follicle stimulating hormone (FSH) or prolactin; Ca^{2+} influx to mast cells; cell culture assays for phagocytosis, lymphocyte activity or TNF release; platelet aggregation activity or activity against HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma cells, antimitotic activity, antiviral activity in infected cells,
15 antibacterial activity (bacterial cell culture) and antifungal activity. Tissue or whole animal assays may also be used including anti-inflammatory mouse ear dermatitis, rat paw swelling; muscle contractility assays; passive cutaneous anaphylaxis; vasodilation assays; or whole animal carbon clearance tests. These assays are available from a variety of sources including Panlabs™ Inc. (Bothell, WA).
20

5.4.3. ANTICANCER ACTIVITY

The anticancer effects of drug can be studied in a variety of cell culture systems; these include mouse leukemias, L 1210, P388, L1578Y etc. Tumor cell lines of
25 human origin like KB, and HeLa have also been used. In a typical assay tumor cells are grown in an appropriate cell culture media like RPMI-1640 containing 10% fetal calf serum. The logarithmically growing cells are treated with different concentrations of test material for 14-72 hours depending upon cell cycle time of the cell line. At the end of the
30 incubation the cell growth is estimated by counting the cell number in untreated and treated groups. The cell viability

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can be ascertained by trypan blue exclusion test or by reduction of tetrazolium dyes by mitochondrial dehydrogenase. The ability of a drug to inhibit cell growth in culture may suggest its possible anticancer effects. These effects can be verified in animals bearing tumors, which are models for
5 human disease (Khawaja, T.A., et al. (1986) *Oncology*, 43 (Suppl. 1): 42-50).

The most economical way to evaluate the anticancer effects of an agent is to study its effects on the growth of tumor cells in minimum essential medium (MEM) containing 10% fetal calf serum. The drug-exposed cells (in duplicates) are
10 incubated in a humidified CO₂ incubator at 37 °C for 2-4 days, depending upon the population-doubling time of the tumor cells. At the end of the incubation period the cells are counted and the degree of cell growth inhibition is calculated from a comparison with untreated controlled cells grown under identical conditions. The type of cell lines
15 used have varied from laboratory to laboratory depending upon individual needs. The National Cancer Institute (NCI) in the United States recommends the use of KB cells (a human nasopharyngeal carcinoma) for the evaluation of anticancer drugs *in vitro*. The cell growth inhibition is determined by
20 estimating the protein content (Lowry's method) of the drug-treated and untreated controls. NCI has also recommended the use of suspension culture of mouse leukemia P388 for the evaluation of anticancer potential of plant extracts and related natural products.

Mouse leukemia L1210 cells, cultured in microtiter
25 plates are routinely used for *in vitro* assays for anticancer activity. The cell population-doubling time of leukemia L1210 is 10-11 h and a drug exposure of 48 h (3-4 generations of logarithmic growth) is used for the evaluation of its antitumor activity. For growth inhibition studies all stock solutions and dilutions are made with sterile 0.9% NaCl
30 solution. The cell cultures are seeded at $2-5 \times 10^4$ cells/ml in duplicates for each inhibitor concentration in a

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microtiter place (0.18 ml/well). The inhibitors are added in 0.02 ml volume to achieve 1:10 dilutions in each case. The covered microtiter plate is incubated for 48 h in a humidified CO₂ incubator containing 5% CO₂ in air. At the end of the incubation period aliquots of each well are added to a
5 measured volume of isotonic saline and counted in an electronic cell counter. The cell viability is determined by trypan blue exclusion. The results are calculated by plotting percent cell growth inhibition (as compared to cell density of the saline-treated controls) versus log of drug concentration and expressed as the concentration which caused
10 50% inhibition in cell growth (IC₅₀) as determined from the graph.

The cytotoxic effects of a drug on a tumor cell line may also be evaluated. However, these experiments require longer periods of time to study and are more expensive. In these studies drug-treated cells are washed free of drug and then
15 plated in soft agar or an appropriate medium and the cellular viability is estimated by the ability of the surviving cells to multiply and form microscopic colonies. The number of cellular colonies obtained with certain drug concentrations is compared with those obtained from untreated controls to evaluate cell kill or cytotoxic activity. In studies with
20 mistletoe extract we have used loosely adherent cultures of EMT-6 cells (a mouse mammary adenocarcinoma). These cells are grown in Eagle's MEM (F14) containing 10% dialyzed fetal calf serum and antibiotics. The cell suspension is spun and the pellet suspended in Spinner's medium supplemented with 10% dialyzed fetal calf serum (70 cells/ml), plated in
25 plastic Petri dishes and incubated for 2 h to permit cells to attach. At this time cells are exposed to various concentrations of extract for 2-24 h. Then, the medium is removed and replaced with drug-free medium and the dishes incubated for 5-7 days. The colonies are stained with methylene blue (0.33% in 0.01% KOH) and counted with an
30 automatic colony counter. The plating efficiency of EMT-6

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cells is 46%. (Khwaja et al., 1986, *Oncology*, 43(Supp. 1):42-50).

5.4.4. ANTIVIRAL ACTIVITY

5 The antiviral activity of different drugs can be ascertained in cell culture of human cell lines like HeLa or H9 lymphoma cells. These cells are infected with virus and the virus is allowed to propagate in cell cultures. The ability of virus to produce cell lysis or cytopathic effects is taken as the end point. For example, HIV infection of H9
10 cells results in production of multinucleated cells. These cytopathic effects, if reduced or eliminated by certain concentrations of the drug, indicates its potential as an anti-HIV agent. These results can be validated by estimation of viral enzyme in the cell cultures, e.g., by studying the amount of the expression of viral reverse transcriptase. A
15 decreased expression of the viral enzyme would support antiviral effect of the drug treatment (Khwaja, T.A. U.S. Patent No. 5,565,200; J. Levy et al. 1984, *Science* 225: 840).

5.5. ANALYTICAL METHODS FOR ANALYZING CHEMICAL COMPONENTS

20 There are many methods to separate and analyze individual chemical components including gas chromatography (GC), mass spectroscopy (MS), GC-MS, high performance liquid chromatography (HPLC), HPLC-MS, thin layer chromatography (TLC), high performance TLC (HPTLC) gel chromatography and reverse phase chromatography (RPC). These chromatographic
25 methods may be performed either on an analytical scale or a preparative scale. To determine the actual chemical structure of unknown components, nuclear magnetic resonance (NMR) and mass spectrum fragmentation analysis are typically used.

The determination of the type of chromatography will
30 depend on the chemical components most likely responsible for the bioactivity. For example if the bioactivity is likely

due to fatty acids, the fatty acids are esterified and the esters analyzed on a GC. For organic compounds with alcohol groups, they are modified to prepare ethers, silyl derivatives or other less polar functional groups. These derivatives are then suitable for analysis by GC (Steinke et al., 1993, *Planta Med.* 59:155-160; Breu et al., 1992, *Arzneim.-Forsch/Drug Res.* 42(1):547-551). If the activity is most likely due to flavonoids, HPLC is the method of choice. Reverse-phase HPLC (RP-HPLC) has been used to analyze flavonoids from a variety of botanicals, specifically hawthorn, passion flower, chamomile, ginkgo (Pietta et al., 1989, *Chromatographia* 27(9/10):509-512). Plant constituents have been quantitatively determined by TLC (Vanhaelen and Vanhaelen-Fastre, 1983, *J. Chromatography* 281:263-271) as well as MS-analysis for garlic. CRC Handbooks of Chromatography on "Analysis of Lipids", K. D. Mukherjee, "Analysis and Characterization of Steroids", H. Lamparczyk, J. Sherma, and "High-Performance Liquid Chromatography of Peptides and Proteins", C.T. Mant and R.S. Hodges, are available and describe columns and solvent systems.

5.6. ANALYSIS OF FRACTIONS

In an alternative embodiment, instead of a fingerprint based on discrete chemical components of known bioactivity, one may also establish the PharmaPrint® using defined fractions or classes of compounds. Some chemical constituents in botanicals form such a complex mixture of closely-related components that, from a practical point of view, it is desirable to base the PharmaPrint® on fractions or classes of components rather than on individual components. Examples of these types of components are lectins (sugar-binding proteins) or glycoproteins as well as the silymarins in milk thistle. There are many examples of fractional analysis (Gel Filtration Principles and Methods

Pharmacia Biotech, Rahms i Lund: Sweden; Utsumi et al., 1987, *J. Biochem.* 101:1199-1208).

5.7. METHODS OF USE OF PHARMAPRINTED™ MATERIALS

5 After the botanical material has an established fingerprint, individual samples are then analyzed to determine if they fall within the accepted standards. Once the sample has been approved it is suitable for a variety of diseases relevant to humans and animals. Such materials are useful in clinical trials so as to provide materials of
10 consistent quality and precise dosage formulations for trials. The PharmaPrinted™ material is also useful for toxicological tests in animals where once again the consistency of the material is useful for quantitative toxicological analysis. In many cases it would be of use as a reference material for analytical or biological use.

15 The PharmaPrinted™ botanical materials are useful for any disease state for which a botanical drug is associated. See for example Leung and Foster, 1996 and Herbal Drugs and Phytopharmaceuticals, 1994. More specific examples of disease states or therapeutic indications include AIDS, adaptogen, mild-to-moderate depression, anti-arthritic, anti-
20 cancer, anti-diarrhetic, anti-helmenthic, anti-inflammatory, anti-nausea via GI, anti-rheumatic, anti-spasmodic, anti-ulcer, angina, antibacterial, antimutagenic, antioxidant, antiviral, arteriosclerosis, arthritis, asthma, blood pressure, benign prostatic hyperplasty (BPH), bronchial asthma, bronchitis, calmative, cough, cerebral circulatory
25 disturbances, cholesterol lowering, cirrhosis, dermatological anti-inflammatory, diabetes, diuretic, drastic cathartic, dysmenorrhea, dyspepsia, emphysema, environmental stress, expectorant, free radical scavenger, GI distress, hemorrhoids, hepatitis, hepatoprotective, hypertension, hyperlipidemia, hyperprolactinemia, immunomodulatory
30 activity, increase fibrinolysis, resistance to bacterial infection, inflammation, insomnia, lactation, liver

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protection, longevity, menstrual cycle regulation, migraine, muscle pain, osteoarthritis, pain, peripheral vascular disease, platelet aggregation, PMS, promote menstrual flow, prostatic disorders, reduce triglycerides, relieve menstrual pain, respiratory tract infections (RTI), retinopathy,
5 sinusitis, rheumatism, sedative, sleep-promoting agent, sore throat, stimulate hair growth, superficial wound healing, tinnitus, topical eczema (dermatitis), urinary tract infection (UTI), varicose veins, venous insufficiency or wound healing.

Other indications include anti-hemorrhagic, anti-
10 microbial, anti-parasitic, anti-pyretic, cardiotonic, carminative, cholagogue, demulcent, diaphoretic, emetic, emmenagogue, emollient, febrifuge, galactagogue, hepatic, hypnotic, laxative, nervine, pectoral, rubefacient, stimulant, tonic, vulnerary, canker sores, pyorrhea, gingivitis, gastritis, ulcers, gallstones, intermittent
15 claudication, cold, influenza, laryngitis, headache, shingles, cystitis, kidney stones, atopic vaginitis, uterine fibroids, osteoporosis and gout.

Garlic is generally reputed to be beneficial in the prevention and treatment of arteriosclerosis and its consequences. A number of clinical studies have also
20 revealed that garlic is effective for reduction of serum cholesterol levels, inhibition of platelet aggregation, and enhancement of blood fibrinolytic activity. Further, garlic has been studied for treatment of hypertension, hypoglycemia, digestive ailments, colds, flu, bronchitis (Foster, 1991, Garlic: *Alium Sativum*, Botanical Series No. 311 American
25 Botanical Counsel, Austin, Texas, pp. 1-7) and is also purported to have antibacterial and antifungal activity (Commission E Monograph, July 6, 1988). Garlic has long been used in folk medicine for treatment of Candida infections, especially of the skin.

A mixture of garlic with vitamin B₁ has been used to
30 protect humans (and animals) against blood-feeding insects. Extracts of crushed garlic cloves have been shown to be

effective in humans (and animals) for combating fleas (Koch et al., 1996, Garlic: The Science and Therapeutic Application of Allium Sativum L. and Related Species, Williams & Wilkins, Baltimore).

Results from a number of studies have indicated that
5 garlic favorably affects various forms of cancer (e.g. Spivak, 1962, *Vopr. Onkol.* 8: 93-96).

5.8. PHARMAPRINT® OF GARLIC

10 5.8.1. BIOLOGICAL PHARMAPRINT®

An exemplary biological PharmaPrint® value, derived using the methods described herein, was determined for garlic using the platelet aggregation factor (PAF) assay. See, *infra*, Section 6.4.2.2. for a detailed discussion and explanation of the PAF assay. The PAF PharmaPrint® value is
15 a range of 20 ± 10 % inhibition upon exposure to garlic extract at 10^{-4} M. Calculations for the extract are based on an assumption of an average molecular weight of 200.

By way of example, a PharmaPrint® based on the bioactivity of extract in the PAF assay, would require bioactivity equivalent to 10-30% inhibition in the PAF assay
20 at 10^{-4} M extract.

In an alternative embodiment, the PharmaPrint® may be developed based on bioactivity equal to or greater than the lower end of the range of bioactivity values observed. As an illustrative example of this embodiment, the PharmaPrint® value based on the bioactivity of total extract
25 in the PAF assay (20 ± 10) would be at least 10% inhibition at 10^{-4} M.

Exemplary biological PharmaPrint® values, derived using the methods described herein, were also determined for garlic using the angiotensin converting enzyme (ACE) assay. These values are presented in Table 1, below. See, *infra*,
30

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Section 6.4.7 for a detailed discussion and explanation of the ACE assay.

TABLE 1. BIOLOGICAL PHARMAPRINT®

5 Bioassay Marker	Ranges (IC ₅₀) µg/mL		
	Broad Range (Mean ± 3 STD)	Medium Range (Mean ± 2 STD)	Preferred Range (Mean ± 1 STD)
10 Angiotensin Converting Enzyme	40.0 - 2000	100 - 1200	400 - 900

5.8.2. CHEMICAL COMPONENTS

A review of the relevant literature relating to garlic reveals the marker compounds illustrated below in
15 Table 2. See, *infra*, Section 6.5 for a detailed discussion and explanation of the selection of the chemical compounds.

TABLE 2A. CHEMICAL COMPONENTS

20	CHEMICAL COMPOUND	% OF EXTRACT (W/W)
	Allicin	0.01 - 0.5
	S-alkyl cysteines	0.001 - 0.10
	gamma-glutamyl cysteines	0.001 - 0.10

TABLE 2B. CHEMICAL PHARMAPRINT®

25	Chemical Marker	Ranges (%) w/w		
		Broad Range (Mean ± 3STD)	Medium Range (Mean ± 2 STD)	Preferred Range (Mean ± 1 STD)
30	Alliin	0.05—3.87	0.1—3.00	0.5—2.12
	Allicin	0.11—1.19	0.29—1.01	0.47—0.83

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	Chemical Marker	Ranges (%) w/w		
		Broad Range (Mean $\pm$ 3STD)	Medium Range (Mean $\pm$ 2 STD)	Preferred Range (Mean $\pm$ 1 STD)
5	Glutamyl-S-allyl-cysteine (GAC)	0.06—5.00	0.60—4.00	0.90—3.00
	Glutamyl-S-trans-1-propenyl-cysteine (GPC)	0.08—2.6	0.66—1.34	0.80—1.21
10	Glutamyl-phenylalanine (GP)	0.06—0.85	0.17—0.63	0.27—0.52
	Total Sulfur	0.10—1.25	0.23—1.10	0.38—0.93

5.8.3. CONVERSION RATIO

PharmaPrint® values developed using dry powdered extracts of a botanical material can be converted to values relevant to dry weight of raw botanical material using the ratios illustrated in Table 3 below. Thus, to convert PharmaPrint® values based on a dry powdered extract to values relevant to a dried plant material, one would divide by the appropriate factor in Table 3.

The ratios shown in Table 3 will be helpful in determining proper amounts of the listed botanical materials to be combined with pharmaceutical grade garlic to produce pharmaceutical grade combinations.

25

30

TABLE 3. CONVERSION RATIOS

CONVERSION RATIOS	
Botanical	Ratio (powder to extract)
5 Saw Palmetto	10:1
St. John's wort	5:1
Valerian	5:1
Echinacea	5:1
Ginkgo	50:1
10 Ginseng	5:1
<i>V. agnus-castus</i>	10:1
Black Cohosh	1:1
Bilberry	100:1
Milk Thistle	40:10

15

The following example is presented for purposes of illustration only and is not intended to limit the scope of the invention in any way.

20

6. EXAMPLE: PHARMAPRINTING™ GARLIC

The following example illustrates development of a PharmaPrint® of garlic.

6.1. COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of garlic. Some of the major products are Kyolic™ (Wakanuga Pharmaceutical Co., Ltd.) (Osaka, Japan)), and Garlinase 4000™ (Enzymatic Therapy (Green Bay, Wisconsin)). Garlic powder tablets are also available from Lichtwer Pharma, GmbH (Germany) and Garlic capsules are available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada). Shaklee, Herbal Choice-Botalia, Nature's

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Way, Sunsource, PhytoPharmica, Lichtwer, Bayer Consumer also supply garlic.

6.2. CLINICAL USE

5 Garlic is generally reputed to be beneficial in the prevention and treatment of arteriosclerosis and its consequences. No known contraindications or interactions with other drugs are associated with the use of garlic. The odor of garlic may pervade the breath and skin. Gastrointestinal symptoms (e.g., heartburn, flatulence, diarrhea) or allergic reactions may occur. Persons taking
10 anticoagulant drugs should consume garlic with caution.

6.2.1. PRIMARY INDICATIONS

A number of clinical studies have revealed that garlic is effective for reduction of serum cholesterol levels, inhibition of platelet aggregation, and enhancement of blood
15 fibrinolytic activity. However, relatively high doses of garlic, i.e., 0.25 to 1.0 g of fresh garlic daily per kg of body weight (5 to 10 average-sized cloves), were employed in most of these studies. Smaller doses (1.8-2.7 g/day) of fresh garlic and powder (0.6-0.9 g/day) appear to be
20 effective in treating hyperlipidemia: a study of 261 patients with total cholesterol and/or triglyceride levels exceeding 200 mg/dL showed that daily administration of 800 mg of garlic powder reduced cholesterol and triglyceride levels by 12 and 17%, respectively (Mader, 1990, *Arzneimittel-Forschung*, 40:1111-1116). The
25 hypocholesterolemic activity of garlic in a rat study was observed to be an effect of the essential oil fraction (Kamanna and Chandrasekhar, 1984, *Indian J. Med. Res.* 79:580-583).

6.2.2. SECONDARY INDICATIONS

30 Garlic has been studied for treatment of hypertension, hypoglycemia, digestive ailments, colds, flu, bronchitis

(Foster, 1991, Garlic: Allium Sativum, Botanical Series No. 311 American Botanical Counsel, Austin, Texas, pp. 1-7) and is also purported to have antibacterial and antifungal activity (Commission E Monograph, July 6, 1988). Garlic has long been used in folk medicine for treatment of Candida
5 infections, especially of the skin.

A mixture of garlic with vitamin B₁ has been used to protect humans (and animals) against blood-feeding insects. Extracts of crushed garlic cloves have been shown to be effective in humans (and animals) for combating fleas (Koch et al., 1996, Garlic: The Science and Therapeutic
10 Application of Allium Sativum L. and Related Species, Williams & Wilkins, Baltimore).

Results from a number of studies have indicated that garlic favorably affects various forms of cancer. In one study, 35 patients with cancers of the lung, cervix, stomach, mammary gland, or larynx were given a garlic juice
15 preparation in doses of 0.2 to 2 mL intravenously or 1 to 5 mL intramuscularly for 3 to 7 days; twenty-six of these patients showed positive treatment results, although no patient showed complete healing (Spivak, 1962, Vopr. Onkol. 8: 93-96).

20

6.3. FRACTIONAL ANALYSIS

The fractional analysis of garlic is performed using supercritical CO₂ chromatography (SFC) or reverse phase chromatography.

The fractional analysis of the chemical components in
25 garlic is performed using column chromatography on normal or reversed phase support media (Michahelles, 1974, Ph.D. Dissertation, Univ. Munich) or supercritical CO₂ chromatography (Calvey et al., 1994, J.Ag. Food Chem., 42:1335-1341). The supercritical chromatography is performed using SFC-FSE™ grade CO₂ (Air Products and Chemicals, Inc.;
30 Allentown, PA) with a 5% phenylmethyl polysiloxane column.

Special chromatographic techniques to isolate garlic constituents such as allicin are thoroughly described in the literature (Thomas and Parkin, 1994, *J. Agric. Food Chem.* 42:1632-1638; Hayashi et al., 1993, *Biosci. Biotech. Biochem.* 57:162-163; Sendl and Wagner, 1991, *Planta Med.* 57:361-362; Blania and Spangenberg, 1990, *Planta Med.* 57:371-375; Iberl et al., 1990, *Plant Med.* 56:320-326; Mochizuki et al., 1988, *J. Chromat.* 455:271-277).

6.4. BIOLOGICAL ACTIVITY ANALYSIS

Garlic has a broad range of biological activities. Its essential oil, water and ethanol extracts inhibit the *in vitro* growth of many bacterial and fungal species. Garlic also has demonstrated antiviral properties (Tsai et al., 1985, *Planta Med.* 460-461) as well as anti-oxidant properties (Yamasaki et al., 1994, *Phytother. Res.* 8:408-412). The volatile oil lowers plasma lipids and extensive *in vitro* and *in vivo* studies have analyzed its impact on lipid metabolism and atherogenesis. Additional cardiovascular improvement mediated by this botanical is through its anti-hypertensive activity. The mechanism of action appears to be due to a direct relaxant effect on smooth muscle. Further, garlic is known to have anti-inflammatory properties and is known to inhibit platelet aggregation.

Values for each bioassay are expressed as a percentage range of inhibition at 10^{-4} M unless otherwise indicated. A percentage inhibition greater than or equal to 20% was deemed biologically significant. Calculations for extracts and fractions are based on an assumption of an average molecular weight of 200.

6.4.1. ACETYL CoA SYNTHETASE

Allicin is the major flavor product of garlic and is enzymatically formed from the cleavage of alliin to allicin

by alliinase (M. Focke et al., 1990, *FEBS Letter* 261:106-108) identified the enzyme acetyl-CoA synthetase as a specific target of allicin. The authors found that allicin inhibits the incorporation of acetate, but not that of acetyl-CoA or malonate, into fatty acids. The inhibition of fatty acid
 5 synthesis and lipid formation is an important protective property of garlic involving the cardiovascular system.

The acetyl CoA synthetase assay utilizes enzyme isolated from yeast. The substrate is [³H] acetic sodium acetate in a reaction buffer consisting of 0.1M glycine-NaOH (9.0 pH), ATP and the compound. Substrate and compound are pre-incubated
 10 together for 5 minutes at 25°C followed by addition of 2 nM coenzyme A for an additional 5 minutes at 25°C. The reaction is terminated by addition of HCl and the remaining substrate is determined by scintillation counting. Test compounds are initially assayed at a 100 μM concentration (Grayson and WestKaemper, 1988, *R.B. Life Sci.* 43:437-444). Reference
 15 literature compounds are listed below.

COMPOUND	IC ₅₀ (μM)
Ethyl- γ -adenosine monophosphate	60
Lovostatin	<100
Orotic Acid	<100

20 6.4.2. ANTIPLATELET ACTIVITY

It has become more certain in recent years that platelets play an important role in the development of atherosclerosis. Endothelial injury leads to the exposure of subendothelial collagen to circulatory blood cells with the result that accumulation of macrophages and platelets takes
 25 place at the site of injury. During this process the platelets secrete many chemicals, including vasoactive substances and platelet-derived growth factor (PDGF). This results in cellular proliferation and migration of smooth muscle cells resulting in growth of the atherosclerotic lesion.

30 The effect on platelet aggregation and platelet synthesis of thromboxane and lipoxigenase products from

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endogenous arachidonic acid was examined by Srivastava and Justesen (1989, *Wein. Klin. Wochenschr.* 101: 293-299) with an aqueous extract of garlic, thin-layer chromatography (TLC) fractions of the extract, and an ether-chloroform extract of the aqueous extract. They found that the aqueous and
 5 ether-chloroform extracts inhibited platelet aggregation induced by all agents. Two prep-TLC fractions inhibited epinephrine- and arachidonic acid-induced aggregation. The *in vitro* effects of adenosin and allicin from garlic were found to inhibit platelet aggregation without effecting
 10 cyclooxygenase and lipoxygenase metabolites of arachidonic acid (Makheja et al., 1990, *Chem. Abst.* 112:574).

6.4.2.1. PLATELET AGGREGATION ASSAYS

Briefly, venous blood obtained from either male or female New Zealand derived albino rabbits weighing 2.5-3.0 kg
 15 is mixed with one-tenth volume of trisodium citrate (0.13 M) and then centrifuged at room temperature for 10 minutes at 220 G. The resultant supernatant is platelet rich plasma (PRP). This is subjected to non-reversible aggregation by 200 μ M sodium arachidonate incubated at 37°C. Aggregation is measured by an optical aggregometer. Test material at a 30 μ M
 20 concentration is incubated for 5 minutes with the PRP to determine the percent inhibition of platelet aggregation. Literature reference standards are listed below. The assay is based on work from Bertele et al. (1983, *Science* 220: 517-519).

	COMPOUND	IC50 (μ M)
25	Aspirin (acetylsalicylic acid)	12
	BM 13,505 (Daltroban)	3.2
	BW-755C	1.2
	CGS 12970	120
	*Indomethacin	0.28
	NDGA	35
	Phenidone	2.6
	Phenylbutazone	28

30 *Indicates standard reference agent used; BW-755C = 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline; CGS-12970 = 3-

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methyl-2-(3-pyridinyl)-1H-indole-1-octanoic acid; NDGA = Nordihydroguaiaretic Acid.

In addition to the use of 200 μ M sodium arachidonate to induce platelet aggregation 5 nM platelet activating factor-acether (PAF-acether) is used (Nunez, D. et al., 1986, *Eur. J. Pharmacol* 123:197-205). Literature reference compounds are listed below:

	COMPOUND	IC ₅₀ (μ M)
	Nectandrin A (BN-52021)	3.3
	CGS-12970	26
	CV-3988	10
10	Kadsurenone (L-651108)	1.7
	L-652731	0.83
	L-659989	0.33
	RP-48740	17
	SRI-63441	1.7
	WEB-2086	0.11

CGS-12970=3-methyl-2-(3-pyridinyl)-1H-indole-1-octanoic acid;
 CV-3988=3-(4-hydroxy-7methoxy-10-OxO-3,5,9-trixa-11-aza-4-phosphanonacos-1-yl)-thiazolinium;
 15 L-652731=2R,5R-di(3,4,5-trimethoxyphenyl).

Other assays described in the literature may also been employed. Kieswetter et al. (1991, *Int'l J. Clin. Pharm, Ther, & Toxic.* 29:151-155) describe an other techniques to
 20 assess thrombocyte aggregation. Another assay for venous insufficiency is clinical indication of the vasodilator inhibitory effects. This is done by the study of contractile response of coronary artery segments to acetylcholine (Bettini et al., 1991, *Fitoterapia* 62(1):15-28).

25

6.4.2.2. PLATELET AGGREGATION FACTOR ASSAY

In this assay garlic extract and fractions are analyzed by measuring the binding of [³H]-platelet activating factor (PAF) to PAF receptors. Platelets of male or female New Zealand derived albino rabbits weighing 2.5-3.0 kg are
 30 prepared in modified Tris-HCl pH 7.5 buffer using standard techniques. A 50 μ g aliquot of membrane is incubated with

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0.4 nM [³H-PAF for 60 minutes at 25°C. Non-specific binding is estimated in the presence of 1 μM PAF. Membranes are filtered and washed 3 times and the filters are counted to determine [³H-PAF specifically bound. Samples are screened at 10 μM. See Hwang et al. (1983, *Biochemistry* 22: 4756-4763) for a description of the assay.

6.4.3. INHIBITION OF CYCLOOXYGENASE ACTIVITY

Arachidonic acid is metabolized to prostaglandins by the enzyme cyclooxygenase-1 or-2. The hormonal effects of prostaglandins include decreasing blood pressure; stimulating contraction of smooth muscle; regulation of inflammation; blood clotting, and the immune response. The literature reference compound for this activity is ajoene (as reviewed by Srivastava et al., 1995, *South African J. of Sci.* 91: 68-77).

The effects of garlic extracts and pure garlic constituents on arachidonic acid metabolism have been studied with respect to platelet aggregation. Leukotrienes, which are formed from arachidonic acid through the action of 5-lipoxygenase, play an important role as mediators of inflammatory response. The table below, from Sendl et al. (1992, *Planta Med.* 58:1-7) shows that chloroform and acetone/chloroform extracts of garlic, containing allicin as the major ingredient, strongly inhibit 5-lipoxygenase *in vitro*.

[5-Lipoxygenase] extract (μg/ml)	acetone/chloroform extract (% inhibition)	chloroform (% inhibition)
9.6	--	100.0 ± 0
4.8	--	88.1 ± 8.3
2.4	--	40.8 ± 13.2
1.9	100.0 ± 0	--
0.96	96.7 ± 4.1	--
0.57	70.5 ± 4.1	--
0.45	20.2 ± 5.8	-

6.4.3.1. CYCLOOXYGENASE-1 ASSAY

Cyclooxygenase-1 (from ram seminal vesicles), 125 units per assay tube, is pre-incubated with 1 mM glutathione (GSH), 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1 minute at 25°C. The reaction is initiated by addition of
 5 arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of trichloroacetic acid (TCA). Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity is determined by reading absorbance at 530 nm (Evans et al., 1987, *Biochem.*
 10 *Pharmac.* 36:2035-2037; Boopathy and Balasubramanian, 1988, *J. Biochem.* 239:371-377).

The following reference compounds are used for the inhibition of cyclooxygenase 1:

	<u>COMPOUNDS</u>	<u>IC₅₀ (μM)</u>
15	aspirin	240
	indomethacin	1.7

Compounds and fractions are screened at an initial concentration of 3×10^{-4} . If an activity of greater than 50% inhibition is observed at 3×10^{-4} , a full dose response curve is carried out.
 20

6.4.3.2. CYCLOOXYGENASE-2 ASSAY

Cyclooxygenase-2 (from sheep placenta), 80 units per assay tube, is pre-incubated with 1 mM GSH, 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1
 25 minute at 25°C. The reaction is initiated by addition of arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of TCA. Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity is determined by reading absorbance at 530 nm (Boopathy and Balasubramanian, 1988; Evans et al. 1987;
 30 O'Sullivan et al., 1992, *Biochem. Biophys. Res. Commun.* 187:1123-1127).

The following reference compounds were used for the inhibition of cyclooxygenase-2:

	<u>COMPOUNDS</u>	<u>IC₅₀ (μM)</u>
	aspirin	660
5	indomethacin	12.4

6.4.4. ANTIBIOTIC ACTIVITY

Allicin is the agent in garlic primarily responsible for the antibacterial and antifungal activity. (Feldberg, R.S. et al., 1988, *Antimicrobial Agents and Chemotherapy* 32: 1763-1768). The bacterial strain studied by Feldberg et al. (1988) was *Salmonella typhimurium* but a variety of other pathogenic bacteria and protozoan parasites could be investigated as well (Mirelman et al., 1987, *J. of Infectious Diseases* 156:243-244).

15 Tynecka and Skwarek (1974, *Farm. Pol.* 30: 531-538) showed that at a dilution of 1:1000, garlic juice completely inhibited the growth of yeast. At dilutions of 1:85,000 to 1:125,000, allicin completely inhibits a variety of Gram-positive (e.g. *Enterococcus faecalis*, *Staphylococcus aureus*) as well as Gram-negative (e.g. *E. coli*, *Salmonella* 20 *typhimurium*) bacteria. The antibiotic activity of 1 mg of allicin equals that of 15 IU of penicillin (10 mg penicillin G), which is approximately 1% of the activity of penicillin (Koch and Lawson, 1996, Garlic: The Science and Therapeutic Application of *Allium sativum* L. and Related Species, Williams and Wilkins, Baltimore). Differences in the *in vitro* 25 antibacterial activity (minimum inhibitory concentration) of garlic-derived sulfur compounds and oils is illustrated in the table below derived from Koch and Lawson (Id).

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	Staphylococcus aureus	E. coli
	(MIC, mg/mL)	(MIC, mg/mL)
Allicin	27	27
Ajoene	55	150
Diallyl tetrasulfide	130	1000
Diallyl trisulfide	250	1900
Diallyl disulfide	900	1900
5 Diallyl sulfide	>2500	>2500
Allyl mercaptan	>4000	>4000
S-Allylcysteine	>4000	>4000
S-Allylmercaptocysteine	2000	>4000
Garlic oil (steam distilled)	80	2000
Garlic oil (ether-incubated)	300	300

values represent the average minimum inhibitory concentration
 10 (i.e. the smaller the value, the greater the activity).

A bioassay for a secondary clinical indication is the
 antibacterial activity associated with the caffeic acid
 derivatives of the extract. The assay will follow standard
 bacterial testing evaluating a panel of common pathogenic
 bacteria routinely assayed in quality control testing
 15 laboratories or in clinical laboratories. Several
 manufacturers have commercial laboratories that routinely
 perform these assays.

6.4.5. ANTICANCER ACTIVITY

Garlic consumption has been epidemiologically associated
 20 with reduced mortality from cancer (as reviewed by Dausch and
 Nixon, 1990, *Preventative Medicine* 19:346-361). Several
 laboratories have reported that pre-incubation of tumor cells
 with garlic or its components renders these cells non-
 tumorigenic. No tumors developed in 75 mice injected
 intraperitoneally with sarcoma 180 tumor cells pre-incubated
 25 with diethyl thiosulphinate prepared by incubating S-ethyl L-
 cysteine sulphoxide with allinase from fresh garlic (more
 stable than allicin). On the other hand, 75 control mice
 injected with saline treated tumor cells died in 16 days (as
 reviewed by Srivastava et al., 1995, *South African J. of Sci.*
 91: 68-77).
 30

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Aqueous garlic extract has been shown to exert anticancer effects on cultured human skin cells and to increase their longevity. In mice, the number of tumor cells injected into the peritoneal cavity was significantly decreased after feeding garlic; in addition, delayed
5 initiation of new tumors was observed, as well as an increase in survival time of the animals by 50% (Koch and Lawson, 1996, Garlic: The Science and Therapeutic Application of *Allium sativum* L. and Related Species, Williams and Wilkins, Baltimore).

10 Components of garlic extract such as S-allylcysteine (SAC) and S-allyl-mercaptocysteine (ASSC) have been demonstrated to have anti-proliferative effects in cell culture (Li et al., 1995, *Oncol. Reports* 2:787-791)

6.4.5.1. ANTICANCER ASSAY

15 There are a number of standard bioassays to assess the anticancer activity of garlic. Such assays may involve the use of whole animals or cancer cell lines. Belman et al. (1989, *J. Biochem. Toxicol.* 4:151-160 describe a typical whole animal assay with garlic components, while a general cancer cell line protocol is described below.

20 A preferred bioassay would involve standard proliferation assays using cancer cell lines (e.g., MCF-7 [breast carcinoma], HeLa [cervical carcinoma], SCC-25 [squamous cell carcinoma], NCI-H446 [lung carcinoma], HL-60 [acute promyelocytic leukemia], Hep G2 [hepatoma], COLO 320 DM [colon cancer line]) incubated with the substances or
25 solvent controls for a maximum of 10 days. At the end of the experiment either cell counts or colony formation will be determined. Cell counts for the compounds will be compared to the control to determine the IC₅₀ for the substances.

30

6.4.6. ANTIHYPERTENSIVE ACTIVITY

The effects of garlic preparations on blood pressure have been investigated in both animal and clinical studies. The hypotensive effect of garlic has been attributed to the vasodilative constituents of garlic. It has been reported
5 that an alcoholic garlic extract (2.5 to 25 mg/kg body weight) caused a decrease in blood pressure of 10 to 50 mm Hg in dogs. An aqueous extract of fresh garlic (15 mg/kg of an extract of 56.6 g garlic in 25 mL saline, intraperitoneally) caused a marked and sustained decrease in blood pressure in normotensive rats (Koch and Lawson, 1996, Garlic: The Science
10 and Therapeutic Application of Allium sativum L. and Related Species, Williams and Wilkins, Baltimore).

6.4.7. ANGIOTENSIN CONVERTING ENZYME ASSAY

To test the ability of garlic extracts and their
15 constituents to inhibit the Angiotensin Converting Enzyme (ACE) the enzyme is partially purified from confluent Human Umbilical Vein Endothelial cells (HUVEC) grown in 24-well plates. The final cellular density is between 10,000 to 20,000 cells per well, after cultivation for 14 days and harvesting using trypsinization. The substrate for the enzyme
20 is [³H] Hyppuryl-GlycylGlycine (Amersham, TRK-806) at a final concentration of 0.9 μ M. The reaction is carried out in 55 mM Hepes/Tris (pH 7.5), 110 mM NaCl and 2 mM CaCl₂. After incubating the reaction for five hours at 37°C the radioactive product is measured using a LS 1701 scintillation counter (Beckman) and Formula 989 scintillation cocktail (Packard)
25 (Hoorn, C. M. and R. A. Roth. Br. J. Pharmacol. 110: 597, 1993).

Reference Compound	IC ₅₀ (nM)
Captopril	0.46 $\pm$ 0.3

6.4.8. HMG-COA REDUCTASE ENZYME ASSAY

To test the ability of garlic extracts and their constituents to inhibit the HMG-CoA reductase enzyme, the gene for it is recombinantly expressed in *Eschericia coli* and partially purified. The substrate for the enzyme is 260 μ M (S) HMG-CoA. After incubating the reaction the (R) mevalonate product is measured spectrophotometrically (Beckman) (Omkumar, R. V. et al. *J. Biol. Chem.* 269: 6810, 1994).

Reference Compound	IC ₅₀ (μ M)
Mevastatin	0.51

6.4.9. POTASSIUM CHANNEL ASSAY

To test the ability of garlic extracts, and their constituents, to inhibit the binding of the radioligand to partially purified potassium channels from rat forebrain membranes, the following assay is done. The ligand used in the reaction is 4 pM of [¹²⁵I]apamin (K_d= 7 pM) and non-specific binding is determined in the presence of 100 nM apamin. The reaction is carried out in 50 mM Tris-HCl (pH 7.4), 0.1% BSA and 5 mM KCl. After incubating the reaction for 60 minutes at 0-4°C it is terminated by rapid filtration through glass filters (Filtermat A, Wallac) and the samples are washed several times with ice-cold Tris-HCl buffer using a Tomtec cell harvester. The amount of specific activity is determined using a Betaplate apparatus (Wallac) using a solid scintillant (MetiLex B/HS, Wallac) (Hugues, M. et al. *J. Biot. Chem.* 257: 2762, 1982).

Reference Compound	IC ₅₀ (pM)
Apamin	13

6.4.10. NITRIC OXIDE BIOASSAYS

The nitric oxide synthase (NOS), constitutive neuronal binding assay with garlic extracts and fractions is done

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using techniques standard in the art (e.g. Michael et al., 1993, *Brit. J. Pharmacol.* 109:287-288).

By way of example, but not of limitation, the NOS binding assay is performed as briefly described below.

Using tissue derived from rat brain membranes, a [³H]-L-
 5 N^G-nitroarginine (NOARG) (55 Ci/mmol) radioligand with a final concentration of 5.0 nM, and NOARG, reactions are carried out in 50 mM TRIS-HCl (pH 7.4) for 60 minutes at 25°C. The reaction is terminated by rapid vacuum filtration onto glass fiber filters. Radioactivity trapped onto the filters is
 10 determined and compared to control values in order to ascertain any interactions of test compound(s) with NOS enzyme assay.

<u>Reference Compounds</u>	<u>IC₅₀ (nM)</u>
NOARG	55.4

15

Assay Characteristics:

K_D (binding affinity): 25nM

A nitric oxide synthetase bioassay can be performed. In the second method, nitric oxide synthetase
 20 activity is measured using mouse macrophages as the enzyme source (Second Messenger, NOS). The inhibition of the activity is measured by an enzymatic reaction where Arginine is converted to NO₂ + NO₃. The amount of enzymatic activity is determined spectrophotometrically. A full description of the method is given by Lowenstein and Snyder (*Cell* 70:705,
 25 1992).

6.4.11. BIOACTIVITY ASSAYS

By way of example, but not by way of limitation, several biological activities of garlic were assayed.

Results from PAF and antimicrobial assays are presented
 30 in Table 4A. Four extracts (GL400, GL401, GL402, and GL403) were bioassayed using the PAF binding assay to determine how

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well garlic extracts antagonize PAF's binding to its receptor. The first extract, GL400, is commercial garlic bulb powder (>1% Aliin), the second extract is a whole fresh bulb of garlic (GL401) and the two remaining substances (GL402 and GL403) are commercially available garlic
 5 tinctures. Results from the assay are shown below in column 2 of Table 4A. The four extracts were also tested in an antimicrobial assay measuring the minimal inhibitory concentration (MIC) for *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans* (see column 3 of Table 4A below).
 10

TABLE 4A. BIOACTIVITY ASSAYS

EXTRACTS	% INHIBITION OF PAF RECEPTOR BINDING	MIC FOR MICROBIAL ASSAY
GL400	20	-
15 GL401	-	-
GL402	-	-
GL403	-	-

Results from ACE, potassium channel, HMG CoA reductase enzyme assays are presented in Table 4B.

20

TABLE 4B. BIOACTIVITY ASSAYS

Sample	ACE IC ₅₀ (mg/ml)	K channel (Ca ⁺⁺ dependent) (% Inhibition @ 1% w/v)	HMG CoA Reductase (% Inhibition @ 10% w/v)
25 Extract Average	0.65 ± 0.23	25 ± 8	NA
Room temp extract	50% @ 1 mg/ml	65% @ 10%w/v	76% @ 10%w/v
Boiling water extract	88% @ 1 mg/ml	96% @ 10%w/v	42% @ 10%w/v

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	Fraction	% Inhibition of ACE @ 0.2 mg/ml	K channel (Ca ⁺⁺ dependent) % Inhibition @ 0.2 mg/ml	HMG CoA Reductase % Inhibition @ 1 mg/ml
5	Prep HPLC (0.0-2.6 min)	IC ₅₀ =0.646 mg/mL	NT	NT
	A1	51	61 (95% @ 1 mg/ml)	NA
	B1	62	NA	39
	C1	76	NA	NA
	D1	82	NA	NA
	E1	65	NA	NA
10	F1	29	NA	30
	II	53	NA	NA
	Reference	ACE IC ₅₀ (mg/ml)	K channel Ca ⁺⁺ dependent) IC ₅₀ (mg/ml)	HMG CoA Reductase (% Inhibition) @ 1mM
15	Aliin	0.652	NA	22
	Glutamyl- phenylalanine	0.09	0.25	NT
	Glutamyl- trans-1 propenyl-L- cysteine	0.031	0.21	NT
20	Glutamyl-S- allyl-L- cysteine	016	0.21	NT
	S-allyl-L- Cysteine	NA	NA	26
25	S-methyl-L- cysteine sulfoxide	NA	NA	13

NT= not tested

NA= not active (less than 20% inhibition at the test concentration).

6.5. CHEMICAL ANALYSIS

The chemical components in garlic include the μ -
 30 glutamyl-cysteines, cysteine sulfoxides (i.e. alliin, etc.),
 the thiosulfinates (i.e. allicin), the ajoenes and various

other sulfur-containing compounds. The analysis is performed using HPLC and/or GC (GC-MS). The HPLC analysis is performed using published procedures (Iberl et al., 1990, *Planta Med.* 56:320-326. The GC analyses are performed using published procedures (Saito et al., 1988, *Eisei Kagaku* 34:536-541; 5 Block et al., 1992, *J. Ag. Food Chem.* 40:2431-2438). The chemical analysis of garlic is also performed using supercritical fluid chromatography-mass spectrometry (Calvey et al., 1994, *J. Chromatogr. Sci.*, 32:93-96.

Garlic contains 0.1-0.36% volatile oil (usually ca. 0.2%), alliin (S-allyl-S-cysteine sulfoxide), S-methyl-L- 10 cysteine sulfoxide, enzymes (e.g. allinase, peroxidase, ATPase, phosphatase, polyphenol oxidase and myrosinase) ajoenes (E,Z-methylajoene, E,Z-ajoene, and dimethyl ajoene) protein (16.8% dry weight basis), carbohydrates (mainly fructans), saponins (including sativoside B1, proto-eriboside B, proto-desgalactotigonin, etc.), minerals (including 15 calcium, phosphorus, potassium, sodium, magnesium, iron, boron, copper, zinc, manganese, chromium, nickel, molybdenum, cobalt, iodine, tellurium, selenium, germanium), vitamins (thiamine, riboflavin, niacin, ascorbic acid, pantothenic acid, vitamins A and E), lipids (including triglycerides, monoglycerides, diglycerides, free fatty acids, phospholipids 20 and glycolipids), sterols (β -sitosterol, campesterol and cholesterol), hydrocarbons, tocopherols, amino acids (common including alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine and cysteine 25 sulfoxides), and others.

The volatile oil contains allicin (diallyldisulfide-S-oxide: diallyl thiosulfinate), allyl disulfide, allylmethyl trisulfide and diallyl trisulfide as the major components, with lesser amounts of diallyl tetrasulfide, allylmethyl tetrasulfide, dimethyl tetrasulfide, dimethyl disulfide, 30 dimethyl trisulfide, allylmethyl sulfide, 2,3,4-trithiapentane, diallyl sulfide, diallyl pentasulfide,

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diallyl hexasulfide, allylmethyl pentasulfide, allylmethyl hexasulfide, dimethyl pentasulfide, dimethyl hexasulfide, allylpropenyl disulfide, allylpropenyl trisulfide, and others. Other volatile compounds present include citral, geraniol, linalool, and α - and β -phellandrene.

5 Prostaglandins A₂ and F_{1 α} were recently isolated from a homogenized garlic extract (Leung and Foster, 1996).

The oil macerated garlic contains mainly 2-vinyl-4H-1,3-dithiin and 3-vinyl-4H-12-dithiin, with lessor amounts of E- and Z-ajoenes, allylmethyl trisulfide, diallyl disulfide and diallyl trisulfide.

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We claim:

1. A method for making a pharmaceutical grade garlic, the method comprising the steps of:

5 separating a representative aliquot of a garlic material, said garlic material comprising a plurality of components, into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

10 comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to determine whether the garlic material is a pharmaceutical grade garlic.

15 2. The method according to claim 1, wherein one or more of the marker fractions contain one active component.

3. The method according to claim 1, wherein the method comprises the additional steps of:

20 determining the amount of the active components in at least one of the marker fractions to provide a quantitative compositional fingerprint of the representative aliquot and

comparing the quantitative compositional fingerprint of the representative aliquot to a quantitative compositional fingerprint standard which has been established for a pharmaceutical grade garlic to determine whether the garlic
25 material is a pharmaceutical grade garlic.

4. The method according to claim 1, wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the garlic material and

30 comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to

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determine whether the garlic material is a pharmaceutical grade garlic.

5. The method according to claim 1, wherein the garlic material is an alcoholic extract.

5

6. The method according to claim 1, wherein the garlic material is an aqueous or organic extract.

7. The method according to claim 1, wherein the garlic material is a supercritical carbon dioxide extract.

10

8. The method according to claim 1, wherein the garlic material is an essential oil.

9. The method according to claim 1, wherein the garlic material is a powdered plant material.

15

10. The method according to claim 1, wherein the garlic material is a homogeneous material.

11. The method according to claim 1, wherein the garlic material is a mixture of plant materials.

20

12. The method according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a cardiovascular disorder.

13. The method according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a dysfunction of platelet aggregation disorder.

25

14. The method according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a disease induced by a microbial organism or a virus.

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15. A method for making a pharmaceutical grade garlic, the method comprising the steps of:

providing a garlic material which comprises a plurality of components which have a given biological activity and wherein each component has a standardized
5 bioactivity profile;

separating a representative aliquot from the garlic material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one of the active components;

measuring the amount of each of the active component(s)
10 present in each of the marker fractions;

calculating the bioactivity of each of the marker fractions based on the amount of each of the active components present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the representative aliquot; and

15 comparing the calculated bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to determine whether the garlic material is a pharmaceutical grade garlic.

20 16. The method for making a pharmaceutical grade garlic according to claim 15 wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the garlic material and

25 comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the garlic material is a pharmaceutical grade garlic.

17. The method according to claim 15, wherein the garlic material is an aqueous or organic extract.

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18. The method according to claim 15, wherein the garlic material is a powdered plant material.

19. The method according to claim 15, wherein the garlic material is a homogeneous material.

5

20. The method according to claim 15, wherein the garlic material is a mixture of plant materials.

21. The method according to claim 15, wherein the bioactivity is indicative of use for treating or ameliorating a cardiovascular disorder.

10

22. The method according to claim 15, wherein the bioactivity is indicative of use for treating or ameliorating a dysfunction of platelet aggregation.

15

23. The method according to claim 15, wherein the bioactivity is indicative of use for treating or ameliorating a disease induced by a microbial organism or a virus.

20

24. The method according to claim 1, 4, 15, or 16, wherein the marker fractions comprise a class of related components.

25. A method for making a pharmaceutical grade garlic, the method comprising the steps of:

25

providing a garlic material which has a given biological activity, said garlic material comprising a plurality of components;

separating a representative aliquot of the garlic material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active class of components;

30

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

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comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade garlic to determine whether the garlic material is a pharmaceutical grade garlic.

5

26. A method for making pharmaceutical grade garlic which comprises determining a total bioactivity of a representative aliquot of a garlic material using a platelet aggregation factor assay and a cylcooxygenase assay and comparing the total bioactivity of the representative aliquot
10 with that of a standard to determine whether the garlic material is a pharmaceutical grade garlic.

27. A method for making a pharmaceutical grade garlic, the method comprising the steps of:

separating a representative aliquot of a garlic
15 material, said garlic material comprising a plurality of components, into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;

determining the amount of the active components in at least one of the marker fractions to provide a quantitative
20 compositional fingerprint of the representative aliquot and

comparing the quantitative compositional fingerprint of the representative aliquot to a quantitative compositional fingerprint standard which has been established for a given pharmaceutical grade garlic to determine whether the garlic material is a pharmaceutical grade garlic.

25

28. A method for making a pharmaceutical grade garlic, the method comprising the steps of:

determining a total bioactivity of a representative aliquot of a garlic material and

comparing the total bioactivity of the representative
30 aliquot with that of a total bioactivity of a standard to

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determine whether the garlic material is a pharmaceutical grade garlic.

29. A pharmaceutical grade garlic made by the method according to claims 1, 15, 25, 26, 27, or 28.

5

30. A pharmaceutical grade garlic made by the method according to claim 1, wherein the marker fractions comprise a class of related components.

10

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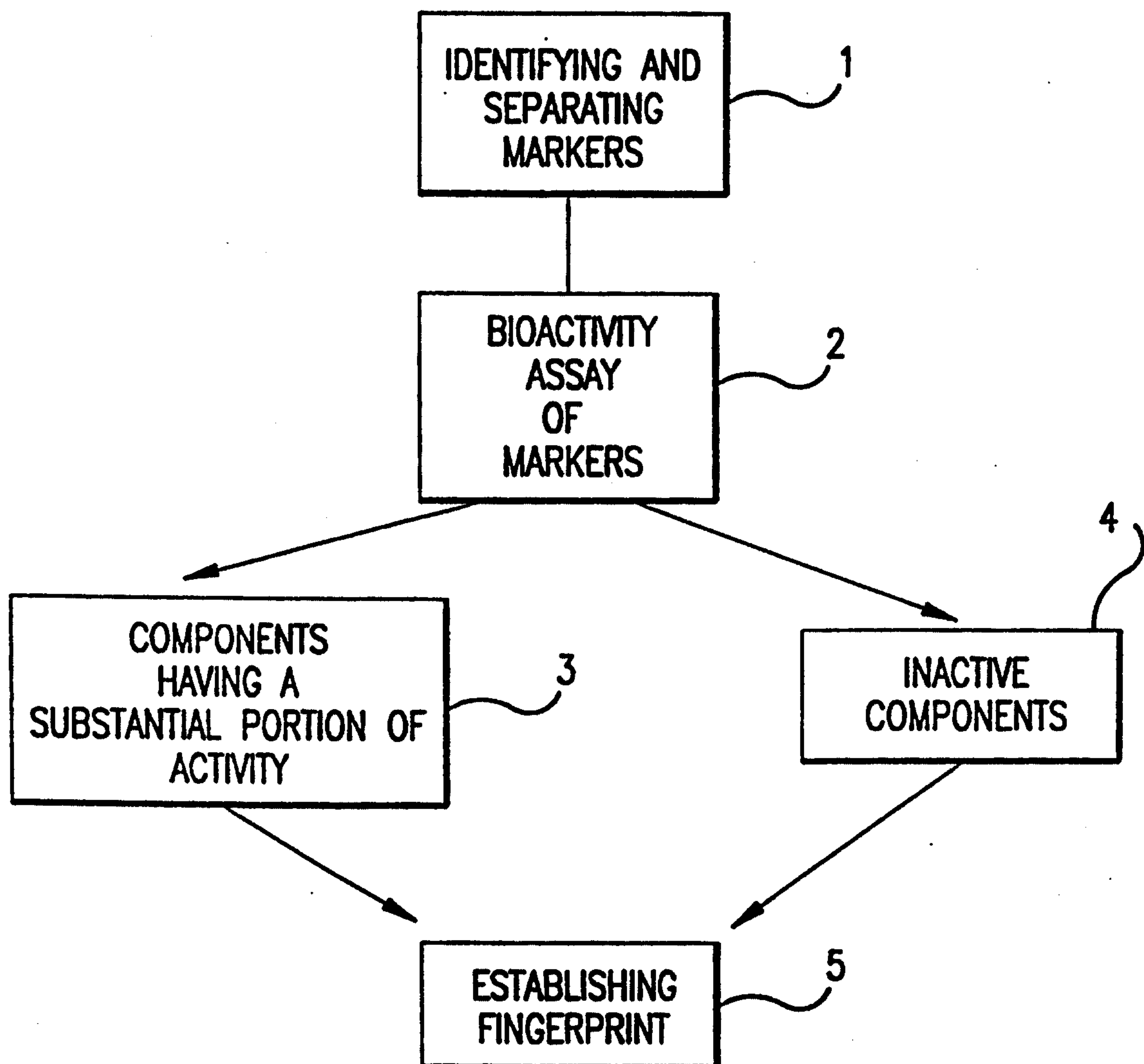


FIG.1

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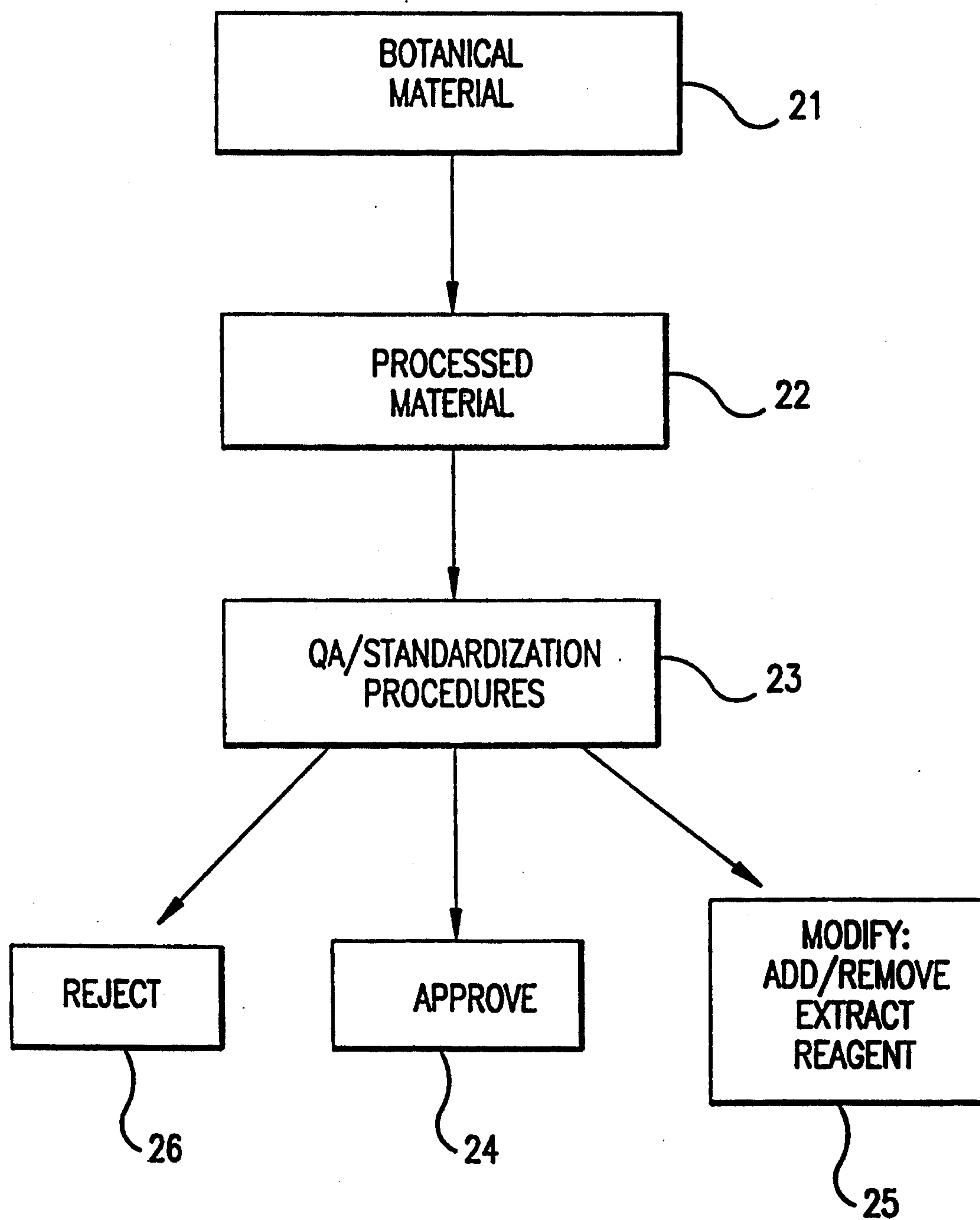


FIG.2

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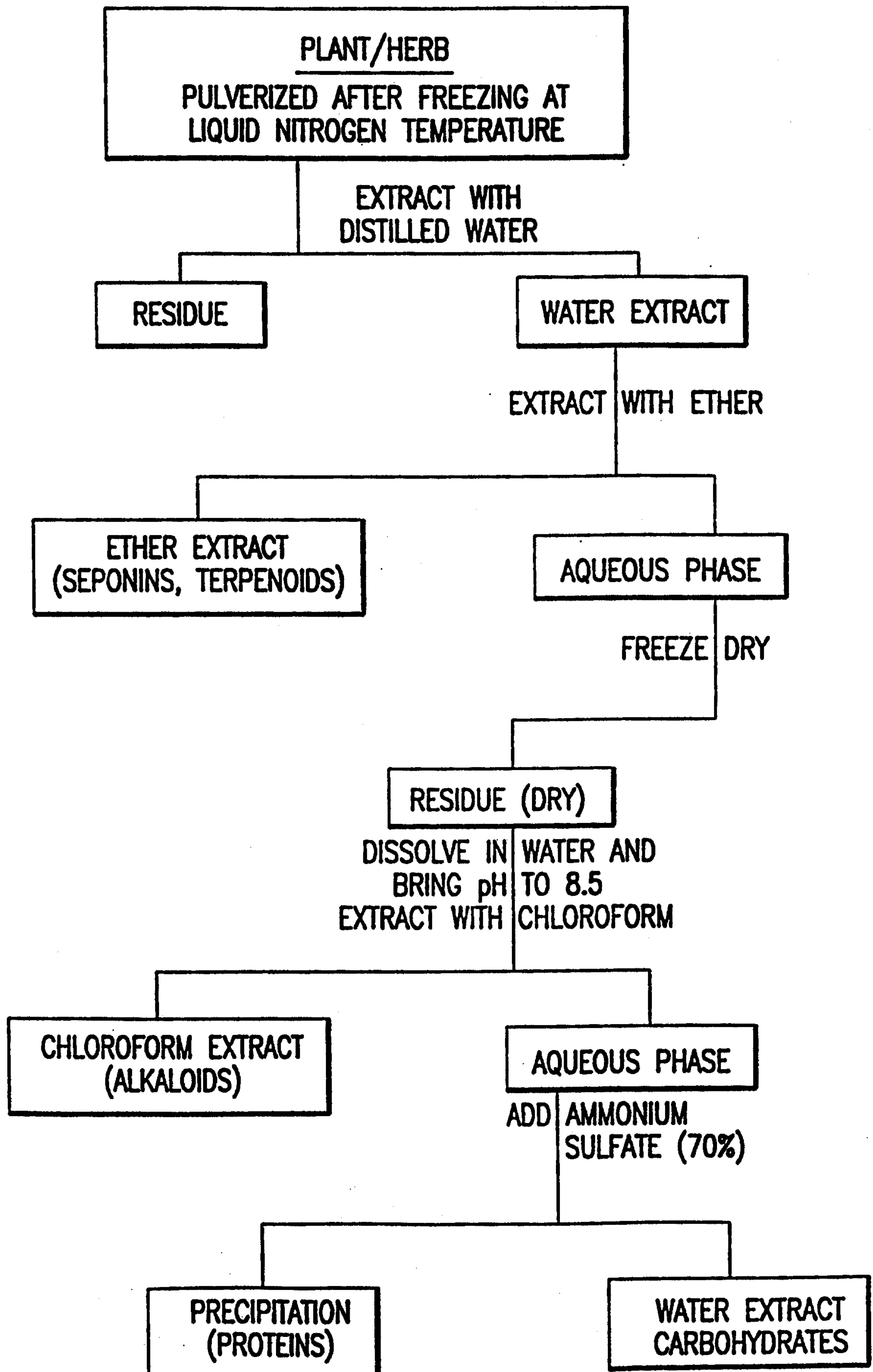


FIG.3