THE SEARCH FOR PLANT SOURCES OF ANTICANCER DRUGS

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Fig. 1. On the way out of the jungles of southwestern Ethiopia with a full load of plant material. The Bonga road is reputedly the worst in Africa.

INTRODUCTION

Plants contain such a vast array of diverse chemical substances that any search of this resource for unusual chemical structures or unique physiological activity can anticipate some degree of success. The search for useful anticancer drugs logically turned to this resource and, over a period of 12 years, has been rewarded with considerable success. Crude extracts prepared from about 1,200 plant species are capable of damaging cancer growth in laboratory animals. The anticancer activity of many of these plants is now known to be due to substances that have no drug potential. But a small though significant number are showing promise as potentially useful drugs of the future.

It would be rash to anticipate that drugs of plant origin will ultimately cure the multiplicity of diseases known as cancer. But we are confident that these products will eventually contribute significantly to the well-being of the afflicted. Indeed, one such drug, vincristine, obtained from the periwinkle (*Vinca rosea* L.), is currently one of the most important drugs used in the treatment of acute childhood leukemia. It is also used in the therapy of some other kinds of malignant disease.

The earliest known record of the use of plants for treating cancer or cancer-like disease is the Ebers papyrus which dates from about 1550 B.C. This early Egyptian work recommended more than 40 plants for treatment of tumors and warts and other possibly malignant growths. Among the plant products recommended were barley, garlic, flax, absinth, coriander, figs, onions, papyrus, dates, and grapes. For most of these, imagination must have played a greater role than efficacy. But it may be more than coincidence that this early work also recommended the berries of juniper, a plant now known to produce a substance that is selectively toxic to cancer cells. The Ebers papyrus also recommended yeast, the source of folic acid which led to

Fig. 2. Collecting bulbs and leaves of *Hymenocallis latifolia* Roem. in the Florida Keys.

use of folic-acid antagonists in cancer therapy. During the subsequent 3,500 years, the use of plants for treating cancer, both in folklore and in medicine, has increased until there are more than 3,000 species now recorded for this use.

The modern search for anticancer drugs began immediately before World War II. War-time success in developing antimalarials and antibiotics encouraged interest in cancer chemotherapy programs by pharmaceutical companies and private research organizations. The major stimulus came in the form of encouragement from the United States Congress, also impressed by war-time achievement in the drug field, which led to the establishment of the Cancer Chemotherapy National Service Center (CCNSC) of the National Cancer Institute in 1955. The present intensive search for anticancer drugs began in 1956 and focused first on synthetic chemicals and fermentation products. From January 1956 through June 1960, more than 110,000 such materials were screened for anticancer activity.

Plant products first entered the program during the Fiscal Year beginning July 1957, and an average of about 5,000 have been tested during each year since 1961.

Screening For Anticancer Activity

Objectives And Philosophy

The objectives of this program are to bring the broad spectrum of chemical substances present in plants before a screen of selected cancer systems in living animals; and, to methodically sift out and identify those of potential value for chemotherapy of cancer in man. Screening can be considered to cover the entire process from collection of plants in the field to the final evaluation of clinical trials of a new drug.

Two logical avenues can be followed in screening plants for any biological activity. One can look first, employing chemical techniques, for classes of phytochemicals such as alkaloids and glycosides that are most likely to produce pharmacological activity. Such substances, once locat-

ed and identified, can then be screened in animal systems for desired activity. This avenue is likely to yield a fairly high number of positive leads, but will not discover physiologically active substances with totally new and unique structures. Or one can, employing laboratory animals, search for plants capable of producing a desired effect, then isolate, identify, and further evaluate responsible chemical compounds. The yield of positive leads is likely to be lower, but the chances of detecting a broader array of pharmacologically active chemical structures are much greater. While the former approach might be appropriate to a short-term program, the latter is better suited to a long-term program, and such broad screening, completely at random, has been followed in the current long-term search for plant sources of anticancer drugs.

Procurement For Primary Screening

Procurement of plant materials for primary screening is conducted primarily by botanists of the Agricultural Research Service, U. S. Department of Agriculture, who since 1960 have supplied almost 19,000 plant samples. Many other individuals or institutions have supplied smaller numbers of plant samples or extracts ready for screening.

Within the USDA, the procurement effort is centered in the Plant Resources Investigations of the New Crops Research Branch (NCRB), formerly Plant Introduction Section. This organization has a tradition of more than 60 years of procurement of plant material for all phases of agricultural, biological, and chemical research.

This long and profitable experience in plant exploration and introduction, resulting in the worldwide procurement of some 500,000 plant collections, has established NCRB as the largest plant procurement agency in the United States. NCRB continues to conduct a traditional plant introduction program. It serves as a worldwide clearing house for plant materials needed not

Fig. 3. Collecting stems of *Dracaena steudneri* in the Bada Buna Forest, near Jimma, Ethiopia.

only by American agriculture, but by agriculturists of many other nations.

The long-term tradition of cooperation with botanical and agricultural institutions in other countries contributes immeasurably to our present program. Contacts abroad permit economical procurement of items of special interest when needs from a foreign area are not sufficient to justify cost of full-scale field projects. These contacts prove helpful to botanical explorers in planning explorations, in making limited field time abroad more effective, and assuring safe and speedy dispatch of collections.

Procurement of plant samples for anticancer screening by USDA botanists was first conducted largely in the continental United States. Field projects were also conducted in Mexico during the early years and some procurement, supported by Public Law 480 funds,[a] was accomplished overseas, especially in Pakistan, Korea, Spain, Yugoslavia, Turkey, Uruguay, and Israel. More recently, procurement by USDA botanists has been increasingly directed to overseas areas including Puerto Rico, Ethiopia, Kenya, Tanzania, and Uganda. Currently, intensive field work is centered in East Africa, with plans to move next to South Africa.

Fig. 4. Ethiopian laborer splitting woody stems of a tree lily, *Dracaena,* to hasten drying.

In addition to collections made directly by USDA botanists, every effort has been made to obtain samples through commercial sources, including suppliers of drug plants, seed, and bulbs. Seed of several hundred plant species are available from domestic and foreign outlets at reasonable cost. Collection of seed by USDA botanists would have been prohibitively expensive; hence, these arrangements with commercial suppliers have permitted the testing of many such materials that would otherwise not be screened. Bulbs of many ornamentals are also available. They have been especially welcome because most are members of families that are well-known sources of pharmacological activity.

It has become evident that field work in the United States is considerably less economical than that conducted overseas. Day-to-day operating costs for a single collector are somewhat less in the United States; but, the daily return of plant material is far greater in many overseas locations, especially in developing countries where vegetation is rich, and where unsophisticated, but effective, labor is abundant and inexpensive. For example, the cost of hiring and supporting one field assistant in the United States will purchase the service of 90 day-laborers in Ethiopia, about 35 in Kenya, or 18 in Mexico. Cost of labor is always a significant part of field expenses, especially when one is collecting large lots of plant materials for isolation of active constituents. The reduced cost of labor more than offsets the somewhat larger overall operating costs in foreign areas, including the extra expense of transporting a botanist overseas. The normally high cost of shipping collections from overseas points to the United States has been offset by negotiation of a special freight-rate classification. This has reduced this cost to an amount comparable to the cost of shipping domestic collections.

Collection of plant samples in the field can be an uncomplicated methodical day-to-day operation; but, in the face of increasing costs and a level budget, innovations must constantly be brought into the field procedure to increase daily production. Over a period of ten years, field techniques have been developed that have raised production from 10-15 collections per day to about 60 samples per day with a return

[a] Under P.L. 480, American agricultural surpluses were sold abroad for foreign currencies, and these currencies were available for supporting research of mutual value to the United States and to the country in question.

Fig. 5. Drying samples of wood and roots at the Kakamega Forest Station, western Kenya.

of as many as 100 samples under exceptionally favorable conditions.

Field experience over this ten-year period has evolved a system that is now in full application in East Africa, the primary center of current field work for this program. The following commentary on procurement is, to a great extent, descriptive of the field work in that region, but it is applicable to other similar areas.

Prior to the initiation of a field project, a long-term plan is laid out so that the collecting will yield the best sampling of the plants of the region (i.e., a maximum number of genera and species with a minimum duplication of species). A first priority area is selected because of its richness and accessibility. Secondary, less accessible areas are selected to complement the first. Selection of the latter may be based on climatic or phytogeographic differences. For example, after intensive collecting in the rich forests and grasslands in the vicinity of Nairobi, Kenya, at an elevation of 5,500 ft. and an annual average rainfall of 34 in., the field effort might move to the upper slopes of Mt. Kenya where, at an elevation of 8-10,000 ft. and annual average rainfall of 70 to 80 in., the species content of the rich forests is quite different. Or the project might move to the Kericho area, where the combination of medium altitude (6,500 ft.) and long rainy season with average annual rainfall of 72 in., produces extremely lush vegetation with many species not seen elsewhere. Other secondary sites might be Kakamega forest in western Kenya, which is the most easterly outlier of the Congo rainforest, or the Usambara Mountains of northeastern Tanzania, which, because of geographic isolation and geologic history, has a flora with a high degree of endemism.

Other factors also enter into selection of field sites. Accessibility by motor vehicle is important because a large volume of plant material is involved. The use of pack animals or porters is not out of the question, but is impractical under most circumstances. A factor that is especially critical is the capability of drying samples in the field. In general, richness and diversity of vegetation are proportional to total annual rainfall. Drying plant samples in a humid environment can be frustrating unless artificial heat is used. The latter is impractical unless volume of collection is greatly reduced or mobility is severely sacrificed.

A principal reason for selection of East Africa for field work was a climate characterized by marked rainy and dry seasons, the former ideal for development of lush vegetation, the latter ideal for conducting field work and drying col-

lections. In some areas the term "dry season" refers to the months when rainfall is at a minimum. Even during the dry period daily showers may occur which, in combination with constant high humidity, make drying difficult. Such areas are often ideal, however, because of rich vegetation. The rich vegetation of these humid areas can often be exploited in regions of uneven topography by locating areas nearby that are in rain shadows and have minimal rainfall and low humidity.

In East Africa the American botanist is accompanied by an African botanist who is a member of the staff of the East African Herbarium (Nairobi) and two African field assistants who, after thorough training, are completely familiar with the required field procedures. In each area where collecting is conducted, a team of local workers is recruited and trained. The collecting proper requires little sophistication, but helpers must learn to follow simple procedures required to assure proper sampling, proper processing of samples, and reliable documentation of the plant materials collected.

These unsophisticated workers have no concept of the importance of correct labeling of collections. They cannot be depended upon to follow logical procedures expected of an educated worker. Methods must be developed that make errors nearly impossible.

Each species encountered is sampled as thoroughly as possible, because some plants concentrate their chemical products in individual organs; for example, in roots, bark, or fruit. Small herbs usually yield a single sample representing the entire plant. Large herbs often yield a separate sample of roots. Trees usually yield separate samples of roots, bark, wood, twigs and leaves, and often a sample of flowers or fruit. All samples are prepared in an amount that, when thoroughly dry, will weigh at least one pound.

Most non-woody plant samples are processed through a portable gasoline-powered compost mill to chop them into small pieces that will dry quickly. Use of the mill was originally intended for chopping samples after drying to reduce their bulk and consequently reduce the high cost, based on volume, of shipping samples to the United States. When field workers noted that almost all types of non-woody plant material pass through the mill without leaving residue to contaminate samples that follow, the mill was used to process fresh samples to hasten drying.

Samples are dried in the sun if the weather is favorable, but covered space is employed for drying during rainy periods. In an environment like that of East Africa with marked wet and dry seasons, field work is scheduled during the dry season, preferably the early months, when vegetation is most luxuriant and drying conditions are at their best.

All collections are carefully documented to identify every sample submitted for screening and to precisely record the location where each was collected. Accurate documentation is essential to permit the return of future collectors to the same location for procurement of duplicate samples of those which demonstrate significant anticancer activity. Each species sampled is assigned a number which is placed (1) on all samples of that species, (2) on notes made at the time of collection which specify and describe the collection locality (including precise map coordinates), and provide a description of the plant, the date collected, and information on local usage, and (3) on dried herbarium specimens collected at the same time. These "voucher" herbarium specimens provide a basis for scientific identification of the plants and a permanent record of each. Five vouchers are collected for each species sampled for deposit in herbaria in Africa, Europe, and the United States.

The dried plant samples are assembled, crated, and shipped by ocean freight to the United States.

All imported plant material passes through USDA's Plant Inspection Station in Washington, D. C. Plant Quarantine Inspectors examine incoming shipments to detect dangerous plant diseases or animal or insect pests. Collections harboring serious pests that could become a threat to American agriculture are fumigated to destroy infestation. After these precautions, the samples are forwarded to a laboratory for preparation of extracts.

Preparation of Plant Extracts

Most extracts for preliminary screening are prepared under CCNSC contract by WARF Institute, Inc., in Madison, Wisconsin. Just before samples are ground in a hammer mill, a few of the larger fragments are removed, placed in a small plastic bag, and labeled with the sample number. If the extract prepared from the sample is active in tests to follow, these fragments will be recovered and compared with the voucher specimen documenting the sample to further

Fig. 6. Processing a large samlpe of *Dracaena steudneri* stems. The side seam of the bag was removed so that the plant material would fall on a tarpaulin.

verify its identity, and compensate for any errors that might have been made in the field or while the samples were being processed for shipping.

After grinding, 100 to 150 grams of the pulverized dry plant material are extracted at room temperature by mechanical mixing in open beakers with 50% aqueous ethanol. After mixing for an hour, the mixture is filtered. Then the alcohol and water are removed from the extract, first by evaporation at mild temperature, then by freeze drying. The dry extracts are bottled and labeled, and forwarded to another laboratory for screening.

The extraction process is designed to remove a broad spectrum of chemical substances from the sample at mild or very low temperature, neither of which is likely to cause breakdown of potentially useful substances. Most of the final extracts are powdery, but some are sticky, gummy, or resinous.

Bio-assay In Laboratory Animals And In Cell Culture

Screening of plant extracts is conducted by nine laboratories working under contracts with the CCNSC. These laboratories currently conduct about 45,000 tests each year with plant products, fermentation products, animal products, and synthetic chemical compounds. About 12,000 tests are devoted to higher plant products. Of these, one-third involve preliminary screening, two-thirds involve testing of fractions and crystalline products.

Plant extracts have been tested against a variety of experimental cancer systems in laboratory animals and in culture. For the screening program to be meaningful, that is, ultimately productive of clinically useful drugs, it must be predictive for anticancer activity in man.

Screening originally employed three cancer systems in mice that were selected after consideration of their response to drugs then in clinical use. Other systems were later introduced, with rats and hamsters as additional hosts, to increase the diversity of the screen in hope of finding additional systems with good predictability for clinical activity.

About three years ago, this broad spectrum of experimental systems was subjected to an intensive evaluation with drugs known to show useful clinical activity in man. This evaluation indicated that a screen of just two systems would predict the activity of almost all drugs of clinical value.

The screen was modified to include lymphoid leukemia L-1210 (LE) in mice and Walker-256 intramuscular rat carcinosarcoma (WM). LE is still regarded as the most useful screening system for predicting clinical activity. It is predictable for cancer in general, not specifically for leukemia. LE is rather insensitive to natural products and very few plant extracts show activity against this system.

Many plant extracts have shown activity against WM. Its use has been discontinued in the primary screen until active constituents can be identified and clinically evaluated to determine whether further use of this system is likely to be profitable. Although it was dropped from the primary screen, WM is still used for fractionation studies to isolate and identify WM-active plant constituents.

Some plants produce potentially useful substances in such minute amounts that they cannot be detected by screening extracts in laboratory animals. The activity of such extracts can sometimes be detected by KB cell culture. When KB activity is concentrated by preliminary chemical fractionation, a substantial proportion of the concentrates show activity in animal test systems. KB cell culture has been used routinely since 1960 and has selected many plant extracts which were inactive in animal test systems. A good example is the activity of *Taxus brevifolia* Nutt.; detected first by KB, it proved active, after concentration, against LE.

Fig. 7. Grinding leaves of a succulent with moka-cha (mortar) and zena-zena (pestle) to hasten drying. These Ethiopian implements are normally used for grinding grain, oilseeds, and spices.

Recently, P-388 leukemia (PS) was added to the screen. It is very similar to LE, but is much more sensitive to known anticancer agents than the latter, and is now being studied as a possible substitute for LE in screening natural products.

Rapidly growing cancers are more sensitive to drugs than are slow-growing cancers. This is probably because a much larger proportion of cells making up the former are in a state of division at any one point in time. These dividing cells are believed to be much more sensitive to drugs than are those that are not dividing. All cancer systems so far used in screening are rapidly growing types. Attention is now being given to selection of an appropriate slow-growing mouse or rat cancer for addition to the screen.

The Walker-256 is a solid cancer implanted in the right hind leg of an albino rat. Lymphoid leukemia L-1210 and P-388 leukemia are cancers of the circulatory system, implanted in the peritoneum of selected strains of hybrid mice bred especially for this program.

In preparation for screening of plant extracts, living cancer cells of the appropriate system are removed from a newly sacrificed animal and implanted in a large number of rats or mice. Some of the animals will serve as controls; that is, their cancer will be allowed to grow without treatment. The other animals are sorted at random into groups of six. The animals in each of these groups will be treated periodically with a crude experimental drug in the form of a plant extract dissolved or suspended in saline solution. Anticancer activity is determined by comparing cancer growth in the treated animals with that in the untreated control animals.

Seven days after implantation, Walker-256 cells in the control animals will have grown to form a solid tumor nearly an inch in diameter. The tumor will weigh between 5 and 7 grams, about 10% of the normal weight of a tumor-free

test animal. A plant extract is considered to demonstrate significant activity if it reduces the mean tumor weight of the six treated animals to less than 42% of the mean tumor weight of the control group.

A leukemia is not a solid tumor and its growth cannot be measured by weight. Activity of plant extracts against LE and PS is determined by comparison of survival time of treated and control animals. Mean survival of untreated control animals varies from 8 to 11 days. Significant activity against LE is reflected by a 25% increase in mean survival time in comparison with the untreated animals of the control group.

KB cell culture was derived from a human cancer of the nasopharynx and is cultured in artificial media in test tubes. Activity against KB is based on the capability of a dilute plant extract (20 micrograms per milliliter or less) to reduce cell growth by 50%. This system is employed as a "pre-screen," that is, to select plant extracts worthy of further testing in animal systems.

Screening experiments are not designed to detect highly spectacular anticancer activity, but to detect activity of a lower order that is significant and reproducible. Evaluation of extracts proceeds and a sequence of up to four independent tests. An extract, active in the initial stage, is tested with a second group of animals. If the original activity is reproduced, a new extract from the same plant sample is prepared and subjected to a third test. Activity at this stage is the basis for preparation of still another extract from the original sample for a fourth and final test. In the first two tests, the extracts are administered to a single group of animals. In the third and fourth tests, the extracts are administered, in a dose-response experiment, to four groups of animals at four dose levels. These doses are double, equal to, one-half of, and one-fourth of the dose level that was acceptable in the second test. Plants that pass the final test are considered "confirmed actives." These become candidates for intensive chemical research to isolate and identify the chemical substance responsible for their anticancer activity.

The testing of a single plant sample may be completed in as little as six months, but may require a year or even two years. Many plants contain highly toxic substances and their extracts kill all of the animals to which they are initially administered. All toxic tests are repeated at a lower dose (half or one-fourth the toxic dose) and testing is continued until a non-toxic, active, or inactive test is achieved. Testing begins at a dose of 500 milligrams of extract per kilogram of animal body weight. When highly toxic substances are present, the final non-toxic dose may be less than one-hundredth of the dose initially administered.

Fig. 8. In warm, sunny areas plant samples can be dried readily in burlap bags. Here, samples are being tied to a rack on top of the field truck to take full advantage of air flow while the vehicle is enroute to the next collecting site. Garfield County, Utah.

The screening program is generating a massive volume of information on the toxicity of plants. Even though the test animals are tumor-bearing, toxicity to these animals correlates well with toxicity to man and livestock. Of thirty species of plants selected at random from a recent review of the poisonous plants of the United States and Canada that were screened for anticancer activity, 13 were highly toxic and 15 were moderately toxic to tumor-bearing mice. An example is the well-known poisonous seed of jequirity bean (*Abrus precatorius* L.). Aqueous extracts of these seed were retested eight times, each at a lower dose, until a non-toxic dose of .006 mg. per kg. of animal weight was reached.

The initial screening of plant samples eliminates about 95% from further consideration. About 5% show enough anticancer activity to justify fractionation, isolation, and identification of active components.

Procurement of Confirmed Active Plant Materials

When the screening of a collection of plants from a single geographic area is complete, a new field project is scheduled for "recollection" of confirmed-active plants from that area. A botanist returns to the original site of collection, at the same season of the year, and obtains a large quantity of the plant that as nearly as possible duplicates the original collection.

Fig. 9. Drying and bagging samples in the attic of an old tea factory near Kericho, Kenya.

We know that plants vary in their chemistry as well as in their morphology. A plant species that produces an active constituent when collected on one soil type in April may not produce that same constituent when growing an another soil type. Or it may produce the same substance but in too small a quantity to be detected by bio-assay. If the recollection is made in September the plant, by then, may have modified the active constituent to a different inactive compound. Genetics, environment, season of collection, and other factors can influence plant chemistry. Consequently, an effort is made to duplicate the original collection as closely as possible. In addition, in order to increase the chance that a new active collection can be made available to the chemist, one or more additional recollections are made at different locations or at different times of the year. In practice, these multiple collections, in lots of 50 lbs. or more, can be made only in areas where abundant labor can be used.

The collector, recognizing that the plant may concentrate the sought-after constituent in one or more organs, will now make a special effort to obtain separate samples representing as many different parts of the plant as possible. This can be a time-consuming task and, again, is often practical only where abundant labor is available.

The preferred size of a recollection has been set at 50 lbs. as an arbitrary figure for lack of knowledge as to what amount is likely to be needed to complete the follow-up chemical work. In some cases a much smaller amount is adequate. In other cases, because of a particularly difficult fractionation procedure that is required, or because the yield of the active constituent is phenomenally low, 300 to 500 lbs. may be required. As we have gained more experience, we are better able to predict to some extent the amount of material likely to be required. For example, we now know that certain tumor systems are sensitive to some types of compounds that have no future as anticancer drugs. We now have some basis for predicting the nature of the activity and can limit collections of such plants to small amounts of as little as 5 lbs., just enough to verify that activity will be due to one of these substances. On the other hand, if the activity is against a tumor system that is not sensitive to these compounds with a low drug potential, and if the active plant is a member of a group that is a known source of pharmacologically active compounds, then a special effort is devoted to the procurement of a minimum 50-lb. sample; and, if conditions permit, a much larger sample is obtained.

Isolation and Characterization of Active Chemical Constituents

The recollections of confirmed active plants are forwarded to laboratories specializing in the chemical fractionation, isolation, and identification of active chemical components. These

plants are being studied in about 35 laboratories, some of which are working with 50 or more species. Some of the larger participants are the Central Drug Research Institute (India), where the work is under the direction of Dr. M. L. Dhar; the Commonwealth Scientific and Industrial Research Organization (Australia), under Dr. C. C. J. Culvenor; the John L. Smith Memorial for Cancer Research in Maywood, New Jersey, under Dr. J. D. Johnston; the Research Triangle Institute, Durham, North Carolina, under Dr. M. E. Wall; the University of Arizona College of Pharmacy, under Dr. J. R. Cole; and the University of Wisconsin School of Pharmacy, under Dr. S. M. Kupchan (now continuing this work at the University of Virginia).

A recollection of a confirmed-active plant must first be tested to determine if it has the same cancer-inhibiting capability as the original active sample. An extract, identical with the first, is tested in a dose response experiment like the second and third tests employed in routine screening. If the new extract duplicates the original anticancer activity, chemists begin a systematic separation of the many and diverse chemical components present in the plant to isolate in pure form the agent responsible for the activity.

Fractionation of a crude extract to isolate a pharmacologically active compound takes advantage of the unique physical and chemical properties of the substances present in the extract. By different chemical and physical techniques, the myriad of substances can be separated into groups or fractions with similar characteristics.

Simple preliminary fractionation is accomplished, for example, by separating a crude extract into non-tannin and tannin fractions by precipitation of the latter with caffeine, or by treating a crude extract with different solvents to dissolve out compounds soluble in each. After each separation the fractions produced are bio-assayed to determine which fraction contains the active agent. Active fractions are then subjected to further separation techniques. Chromatography, which relies largely on the differential adsorptive power of an adsorbent for different chemical substances, is one of the most powerful of such techniques that are available to the chemist involved in isolation of natural products. It is capable of separating substances with the most subtle differences in their physical properties.

Isolation of pure crystalline active compounds is rarely a simple task and may be extremely complex. Isolation of one or two active compounds from a typical plant source frequently requires the separation of the crude extract into about 50 fractions. The isolation of a large number of compounds from a source plant may require 500 or more separate fractions, each of which must be bio-assayed. An active constituent may amount to as little as 0.01% of the dry weight of a source plant.

Once a pure crystalline compound is isolated, its chemical structure must be determined and the task of the chemist may become extremely difficult. Chemical characterization is essential for several reasons. First, the compound may be identical with an active material obtained from another source, and duplicate effort in further evaluation of the compound may be avoided. Secondly, once the structure is known, the chemist can subject the material to techniques that may accomplish minor modifications in its chemical structure that may increase activity or solubility, improve therapeutic index (ratio between the maximum dose that is tolerated and the minimum dose that is effective), or decrease unde-

Fig. 10. Drying and bagging samples at the Kakamega Forest Station. Voucher specimens are in plant presses at left.

sirable side effects. Thirdly, a judgment of the feasibility of its synthesis can be made, possibly leading to its economical production on a large scale. Finally, it is important to be able to recognize the relationship between the compound's biological activity and its chemical structure. Once the structural features responsible for the activity are known, the synthetic chemist can design synthesis of totally new compounds combining these features with other basic types of molecules to develop new active structures.

Preliminary clues to the nature of the compound are provided by standard chemical analysis to determine the elements present and their proportion; by qualitative tests for alkaloids, glycosides, steroids, and other classes of compounds; and by the avenues followed during the isolation and purification of the compound. Chemical characterization of the compound may be fairly simple if it was previously described and an authentic sample is available for comparison by physical methods like melting point, optical rotation, and infra-red absorption spectrum. If the compound is new, and especially if it represents a new or unusual class, highly complex and sophisticated techniques may be required. Mass spectrometry, nuclear magnetic resonance, and single crystal X-ray analysis, among other techniques, determine the number of each kind of atom in the molecule and the position of each in relation to the others.

Preclinical Pharmacology

After a pure active compound is isolated and, sometimes, even while its structure is being determined, the potential new drug is tested in a broad array of animal tumor systems, to determine its spectrum of activity and to gain as much additional information as possible. "Schedule dependency studies" are instituted to determine the effect of different doses, and frequencies and routes of administration. All information about the compound is then reviewed by committee to reach a decision as to whether further evaluation is justified. Many factors are considered: Is the substance active against animal systems that are sufficiently predictive for clinical activity in man? Does it have a sufficient therapeutic index to suggest it may be safe for clinical use? Does it represent a new kind of structure never before evaluated, or is it just one more of a class already well represented by compounds previously evaluated or under consideration? If the compound meets the necessary criteria, it is approved for preclinical pharmacology.

Predictions as to many of a new drug's possible adverse side effects when used clinically (i.e., nature and degree of toxicity or organ damage) can be based on tests in dogs and other animals. This is the primary objective of preclinical pharmacology. These studies are also geared to determine appropriate starting doses applicable to administration of the drug to human patients, the most suitable vehicle, and the most acceptable route of administration. Drugs that meet the necessary criteria are cleared for preliminary clinical trial in human patients.

Large Quantities of Drugs Required For Clinical Trial

Before clinical trial can begin, an adequate supply of the new drug must be available to assure that the preliminary clinical evaluation of the drug can be carried through to a significant conclusion. In some cases the supply situation is so critical that a special procurement effort must be undertaken in order to supply adequate amounts for preclinical research.

Several procurement avenues are open to obtain an adequate supply, and all are carefully compared to determine which is the least costly in terms of both time and financial resources. Some such compounds occur in the plant, or in another species, in sufficient abundance that they can be isolated from their natural source. Such was the case of the new drug lapachol. Its activity was detected by screening the roots of an Indian tree, in which it is present in small amounts. A review of the literature revealed that it was much more abundant in lapacho wood (*Tabebuia* spp.) of Central and South America. Other compounds occur in such minute amounts in the plant source that synthesis, when possible, is likely to be the most economical source. Such is the case of camptothecin, a promising new alkaloid of *Camptotheca acuminata* Decaisne. It appears that a practical synthesis of camptothecin is likely to be accomplished, and an intensive effort is now being made to produce it synthetically.

Still other compounds appear to be difficult of ready synthesis with the knowledge and techniques now available and will probably have to be isolated from the natural source. Harringtonine, a new alkaloid isolated from *Cephalotaxus harringtonia* (Forbes) K. Koch, and now cleared for preclinical pharmacology, is a good example. Fortunately, the substance occurs in the plant in fairly large amounts though the plant grows so slowly that production of adequate amounts of raw material may prove costly.

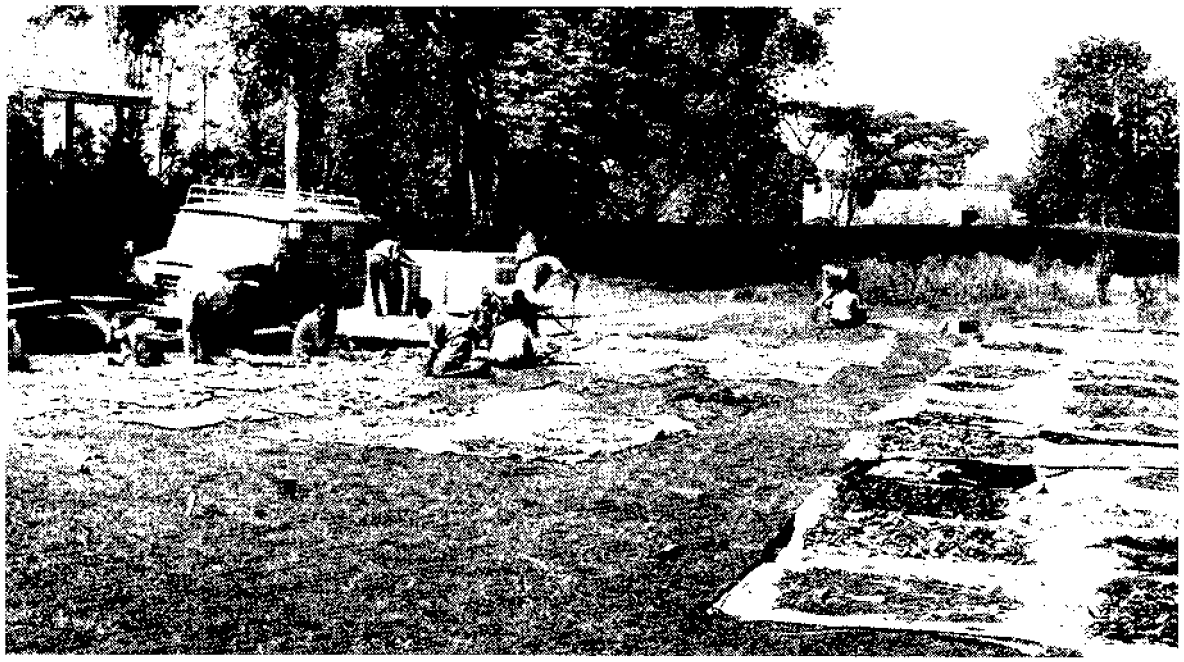

Fig. 11. Drying plant samples at the Forest Station, Londiani, Kenya.

Search For Better Sources of Drugs That Cannot Be Economically Synthesized

When large recollections of confirmed-active plants are prepared, the plants are routinely separated into as many samples as possible. Subsequent bio-assays of these fractional samples will indicate which part of the plant is the best source of the activity. Later sampling, as more material is needed, is designed to contribute as much new information as possible. Little additional effort can be devoted routinely to most of the active plants, for their number is large and most are active against tumor systems with relatively low clinical predictability.

Some of the active plants produce potential new drugs that will be impractical to synthesize. These merit special attention to improve future sources of supply, especially if they are active against LE or, if active against a system of lower priority, they have reached an advanced research stage and appear destined for clinical trial.

The anticancer alkaloid thalicarpine was isolated from roots of *Thalictrum dasycarpum* Fisch. & Lall., where it is present in a small amount. It does not occur in the stems or leaves in an amount sufficient to justify isolation. The plant is abundant in Wisconsin and nearby states. If the alkaloid becomes a useful drug, the quantity of roots needed will be so great that the plant will soon become rare. The fruit produce several times as much alkaloid as the roots. This perennial produces a heavy crop of fruit; it is evident that, over a period of years, a planting or natural stand will produce as much fruit as it will roots.

A large supply of *Thalictrum* seed was obtained from natural stands during the 1969 field season and preliminary research to establish a crop will be initiated in 1970. The crop potential of this plant is enhanced by its production of a seed oil with potential large-scale industrial use.

The active agent taxol was isolated from bark of Pacific Yew (*Taxus brevifolia* Nutt.). Bio-assay of a series of bark samples from different locations from Idaho to California, north to Alaska, indicated that yield of taxol fluctuated considerably. The bio-assay data provided a valuable guide when large-scale procurement was undertaken to provide 2,500 lbs. of bark for isolation of a large supply of the drug.

Assay of other samples of *T. brevifolia* indicated that taxol does not occur in detectable amounts in other parts of the plant. This yew is a small, slow-growing tree. It is abundant but the

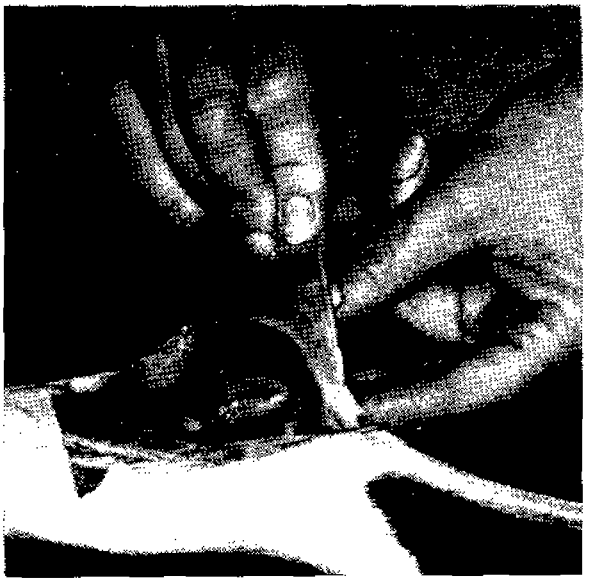

Fig. 12. The animal for the Walker 256 test, the non-inbred albino rat, is injected in the right hind leg with 0.2 cc of a suspension of Walker cells. Ten to 12 days later the tumor usually weighs 5 to 7 grams.

bark will not be a suitable source because the yield of the drug is very low. During the past few years samples of all yew species have been asayed. No data are available to compare yields of taxol but it is evident that all species produce this compound. Should a large-scale need for this substance develop, an intensive selection and breeding program could be directed toward development of faster-growing higher-yielding types.

Extracts of *Camptotheca acuminata* demonstrated confirmed activity against LE in 1962. Intensive chemical and biological research has been focused on this plant since that time. Camptothecin, the principal active alkaloid of this species, is now in preliminary stages of clinical trial.

The tree was introduced from China. In 1962, it was rare in the United States, the total supply amounting to less than 30 specimen trees, most of which were not more than about 15 years old. A small-scale research and production program was undertaken at the USDA Plant Introduction Station, Chico, California, in late 1963. Both research and production expanded gradually during the next few years.

When the structure of camptothecin became known and it was evident that a practical synthesis was likely, agricultural research tapered off. But production has been greatly expanded and, by the spring of 1970, about 15,000 plants will be established in field plantings. These plantings will provide a supply of camptothecin for clinical research until the natural source is replaced by a practical synthesis.

Current agricultural research is minimal but production plantings are under close observation, to detect possible insect or disease problems, and to select individual plants with exceptional vigor or that are good producers of seed. During the spring of 1969, a single seedling with exceptional vigor was selected from a population of 5,000.

Camptotheca grows rapidly and can be adapted to production as a crop if synthesis eventually proves impractical. During August and September 1969, 5,800 lbs. of roots and stems were harvested to provide more of the drug for clinical research.

Plants were removed from alternate rows of two plantings of different age. It appears that the reduced competition will spur growth of the remaining plants to such a degree that the total amount available from these plantings will be at least as great a year later as the amount present during the recent harvest.

In the case of some active plants, a combination of unfavorable factors makes the original plant hopeless as a source of the drug. Such is the case with *Holacantha emoryi* Gray, native to deserts of southern California, Arizona, and northern Mexico. The plant is uncommon and its active constituent is present in very small amounts. Though the plant might grow more rapidly under irrigation, it appears to be a very slow-growing shrub.

The chemical literature, however, reveals that seed of another plant produce significant amounts of a substance that can be chemically converted to the active compound isolated from *Holacantha*. The seed of this plant are used industrially as a source of edible oil and will assure an economical source of the potential new drug.

Clinical Trial

Clinical research is a medical matter beyond the scope of this discussion. It should be noted, however, that the efficacy and safety of a new drug must be demonstrated in human patients before it can be accepted for general use.

Five to 10 new drugs are accepted for clinical trial each year. So far, these have been largely synthetic or fermentation products. Six drugs of plant origin have reached this stage.

A drug is acceptable for clinical trial only after preclinical research establishes that there is a reasonable promise that it will be beneficial to the patient, and that any possible adverse side effects can be countered by known medical means or will be offset by an overall improvement in the patient's well-being. Testing of a new drug at this stage is limited to patients with advanced disease.

Classes of Active Chemical Agents

The search for anticancer agents turned to plants because of the known chemical diversity of this resource. It was hoped that this quest would not only yield a diversity of plant products of potential drug value, but would also provide clues to new types of pharmacologically active chemical structures, and thus broaden the opportunities of chemists attempting to synthesize a broader array of substances with anticancer activity.

This search has been rewarded with numerous new pharmacologically active substances of great chemical diversity. Many are novel chemical types. Most would not otherwise have been available for consideration, either because they are difficult to synthesize, or because, lacking prior knowledge of their potential activity, there would be no logical reason to attempt synthesis.

The discovery of a new class of chemical compounds capable of inhibiting cancer growth is of great value even though the initial representative compounds may prove unsuitable for clinical trial. Each such discovery offers the promise that other more suitable representatives of the class will be isolated from other plants, or result from synthesis or chemical modification of the plant product.

The potential drug value of the classes of compounds discussed below varies from those with little if any future to some with considerable promise. Compounds representing one of the latter groups appear better than did certain clinically established drugs when they were at the same preliminary stage of development.

Alkaloids. The most promising group of new anticancer agents, in number and variety, is the alkaloids. These substances are widely distributed in the plant kingdom though many are concentrated in such families as Apocynaceae, Papaveraceae, Leguminosae, and Ranunculaceae. As a group, they are well-known for their pharmacological activity and play an important role in modern medicine. They are very diverse, indeed so diverse that they defy simple definition. Collectively, the alkaloids are active in a broad spectrum of animal tumors; several are active against LE.

Cucurbitacins. These are highly toxic substances, isolated largely from species of Cucurbitaceae. Recently, they were also isolated from

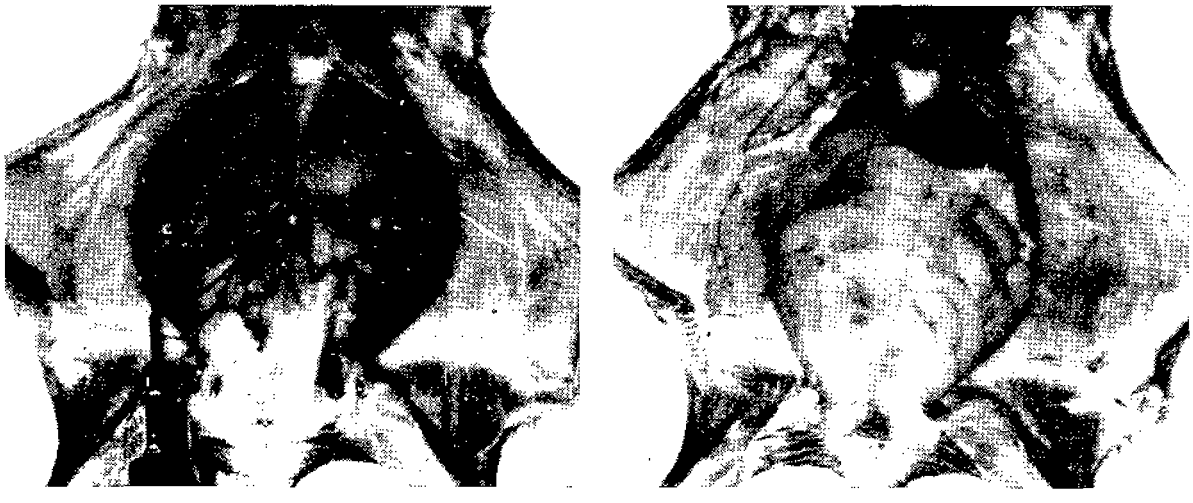

Fig. 13. The L-1210 test system uses strains of especially bred mice. The photos above show the contrast between the distended, darkened abdomen of the leukemic animal from which fluid is being removed with a syringe (left) and the normal mouse (right). The fluid will be diluted for use in other tests.

species of Cruciferae, Scrophulariaceae, and Begoniaceae. These compounds are highly active in KB but have little *in vivo* activity, and, hence, offer little promise.

Diepoxides. Crotepoxide, isolated from the Ethiopian tree *Croton macrostachys* Hochst., is an unusual member of this group of compounds which is represented by a considerable number of synthetics that have shown good activity. Crotepoxide does not have sufficient activty to justify further evaluation, but it is of importance because it is the first diepoxide with anticancer activity to be found in a plant. This suggests that other more suitable compounds of this type may be found.

Digitaloid glycosides and their aglycones. A large number of compounds of this group, including cardenolides, bufadienolides, and withaferins, have been active, especially against KB. A few show activity against *in vivo* tumor systems, but their therapeutic indices are too low to justify further evaluation. This class of compounds is of considerable interest, nevertheless, because it is abundant in nature and few members have been considered.

Lignans. The activity of a large number of extracts from such distantly related genera as *Juniperus, Podophyllum,* and *Bursera,* all active in KB, has proved to be due to lignans. These substances had been considered to have no promise because they are active largely in KB and show only very low activity in *in vivo* tumors. More recently, two new lignans were isolated from a species of *Bursera* which are active in WM. One of them looks sufficiently interesting to justify further evaluation.

Phytosterols. Ligroin extracts from a considerable number of plants were active against WM. Fractionation established that activity was due to sterols, frequently to beta-sitosterol, a substance almost ubiquitous in the plant kingdom. An effort was made to develop this compound, but beta-sitosterol and its active derivatives are unsuitable because of poor solubility and low therapeutic index. The phytosterols are presently of little interest.

Proteins. Proteinaceous materials have been isolated as the active cancer-inhibiting constituents of a considerable number of plants. One, isolated from seed of *Caesalpinia gilliesii* (Hook.) Wall. ex Loefgren, is scheduled for preclinical pharmacology. It was selected from several such materials available because it shows very good activity against WM, and was available in purest form and in adequate amount.

Quinones. Several quinones have been active. The most notable is lapachol, now in clinical trial. It was isolated in small amounts as the active constituent of roots of the Indian tree *Stereospermum suaveolens* DC. A better source is lapacho wood (*Tabebuia* spp.) of tropical America in which it is comparatively abundant. In Brazil, a tea prepared from *Tabebuia* spp. is widely used both by physicians and in folk medicine for treating cancer. This compound is unusual in that it is more active when administered orally than when administered intraperitoneally to experimental animals.

Saponins. These compounds are also widely dispersed in the plant kingdom. A considerable number have shown activity, especially against WM. The most interesting is *Acer* saponin P, which has the largest therapeutic index in WM of any active compound in this group. It is undergoing further investigation and will probably be considered for clinical trial.

Sesquiterpene lactones. These active compounds were isolated largely from plants of the family Compositae. Much of the activity of plants of this family appears to be due to this class of compounds. Most of the active sesquiterpene lactones are active only against KB; a few are active against WM but have a therapeutic index too low to justify further study.

Tannins. These substances are very widely distributed in higher plants, especially in the woody species, and are responsible for the anticancer activity of many crude extracts. They are active especially against WM; only rarely are they active against KB. Tannins are consistently highly toxic with low therapeutic indices. Since they are also difficult to purify and are chemically unstable, they are considered to have little if any promise. Methods are followed early in the fractionation procedure to identify plants with activity due to tannins, and such plants are dropped from the program.

The following tables list anticancer agents isolated from plants that have reached preclinical pharmacology or a more advanced stage and are still under consideration for further evaluation.

AGENTS APPROVED FOR OR NOW UNDERGOING PRECLINICAL PHARMACOLOGICAL EVALUATION

Agent	Class	Source Plant	Origin of Plant
harringtonine	alkaloid	Cephalotaxus harringtonia (Forbes) K. Koch (Cephalotaxaceae)	horticultural sources in U.S. and Italy (native to Japan)
ellipticine	alkaloid	Excavatia coccinea Markgraf (Apocynaceae)	New Guinea
		Ochrosia moorei F. v. Muell. (Apocynaceae)	Australia
dl-tetrandrine	alkaloid	Stephania hernandifolia Walp. (Menispermaceae)	India
d-tetrandrine	alkaloid	Cyclea peltata Hook. f. & Thoms. (Menispermaceae)	India
Acer saponin P	saponin	Acer negundo L. (Aceraceae)	Wisconsin and Utah
uncharacterized protein	protein	Caesalpinia gilliesii (Hook.) Wall. ex Loefgren (Leguminosae)	Arizona

Distribution of Anticancer Activity In Higher Plants

During the past 12 years, about 45,000 crude extracts of higher plants have been screened. About 1,400, representing some 1,200 species, have shown reproducible activity in one or more of the tumor systems used in the screen. The active species represent 158 families.

It soon became evident that there were concentrations of activity in some families, but the broad spectrum of activity (in 158 of 270 families represented) was puzzling. It is now evident it is due to the sensitivity of the tumor test systems to a large number of classes of chemical compounds, some of which are very common and others of which are almost ubiquitous in the plant world.

Active constituents have been isolated from some 240 species. The activity of 35% of these plants is due to tannins; the activity of 10% is due to phytosterols; and the activity of 55% is due to other classes of compounds.

It is evident that, because of the large proportion of activity due to substances with little if any

AGENTS APPROVED FOR CLINICAL EVALUATION

Agent	Class	Source Plant	Origin of Plant
thalicarpine	alkaloid	Thalictrum dasycarpum Fisch. & Lall. (Ranunculaceae)	Wisconsin
acronycine	alkaloid	Acronychia baueri Schott. (Rutaceae)	Australia

AGENTS NOW UNDERGOING CLINICAL EVALUATION

Agent	Class	Source Plant	Origin of Plant
camptothecin	alkaloid	Camptotheca acuminata Decaisne (Nyssaceae)	botanical gardens in U.S. and Taiwan (native to China)
lapachol	quinone	Stereospermum suaveolens DC. (Bignoniaceae)	India
		Tabebuia spp. (Bignoniaceae)	Central and South America
emetine	alkaloid	Cephaelis ipecacuanha (Brot.) Rich. (Rubiaceae)	Central and South America

potential drug value, the distribution of general anticancer activity in higher plants is largely academic. But the distribution of potentially useful activity, due to compounds other than tannins and phytosterols in particular, is of considerable practical importance.

When screening demonstrates that a family or other group is a rich source of activity, the question now arises as to whether or not the activity is due to potentially useful compounds. We are beginning to reach the point where we can predict with some confidence that certain families are very unlikely to yield useful drugs, while others can be recognized as especially promising. Currently, routine procurement is still conducted completely at random, and no family is excluded. But leads to promising sources of activity are being followed up with intensive procurement efforts to locate additional genera and species of impressively active groups.

The anticancer activity of the first 10,000 samples procured by USDA was reviewed to establish guidelines that might be applicable to future procurement. These samples represented 5,478 species in 2,073 genera and 206 families; 11% of the species tested were active and 20% of the genera had at least one active species.

The conifers are the richest source with activity shown by 34% of the species and 57% of the genera tested. Active agents have been isolated from less than half of the active species, but already this group has yielded a considerable diversity of active agents. One is expected to represent a class of chemical compounds that is unique in the program. The most promising are the LE-active alkaloids of *Cephalotaxus* and *Taxus*.

Activity of plants of the order Magnoliales has also been impressive. Twenty-three percent of the species tested were active and 30% of the genera tested were represented by at least one active species. Most of the anticancer agents isolated from this group are alkaloids. The Magnoliales are a well-known source of alkaloids; this, in combination with a good concentration of KB activity in this order, suggests that the activity of many other species is most likely due to alkaloids and unlikely to be due to tannins or phytosterols.

A family with many active species is the Liliaceae. We have screened about 70 of the 250 genera, and 25 genera now have at least one active species. Few active agents have been identified from this group but the family is a well-known source of alkaloids.

Some other promising families with high levels of activity in which the activity appears to be due to substances other than tannins and phytosterols are: Asclepiadaceae, Apocynaceae, Solanaceae, and Ranunculaceae.

Other families have an impressive number of active genera and species, but appear to have little promise as sources of useful drugs because of the nature of the agents responsible for the activity. The activity of almost all species of Cornaceae, Ericaceae, Fagaceae, Onagraceae, Polygonaceae, and Rosaceae is due to tannins. The activity of the Cucurbitaceae is due primarily to the cucurbitacins. The yield of active species of Compositae is equivalent to the average for all families, but the number of active species is high because of the large number of species of this family included in the program. A large proportion of the activity in this family is due to sesquiterpene lactones.

In summation, it is evident that the search for plant sources of anticancer drugs must be a long-term program. It was only recently that a sufficient number of plants had been screened, and enough had been learned about the chemical structure of their active components, that new avenues can be opened, leading to an even more profitable program. Concomitantly, clinical experience, largely with synthetic compounds, has provided valuable feedback in that it has pointed out the experimental tumor systems with greatest clinical predictability and has permitted improvements in the screen. This process of developing a truly predictive screen is at the heart of the whole effort to produce useful anticancer drugs and is continuing. As the screen improves, the nature of the isolated compounds of greatest interest is likely to change, and an increase in the proportion of useful compounds is to be expected.

A long-term research effort, directed toward a specific but distant goal, must be evaluated at any point in time by what has been accomplished in relation to what is reasonably expected at each stage. The search for anticancer agents of plant origin has so far been judged successful at each stage through which it has passed. The ultimate judgment must await the final clinical evaluation of drugs headed in that direction.

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