CHAPTER 4

DISCUSSION

Investigation by *in vitro* methods of antibacterial properties of a substance biosynthesized by plants does not prove that compound to be of therapeutic value. However, *in vitro* techniques to provide a rapid method for detecting antibacterial substances which may warrant more intensive investigation (34).

Antibiotic susceptibility testing involves relatively complex systems in which variation of a number of different components or conditions may influence results. Therefore, control of the reproducibility of the system involves testing microorganisms of known behavior. In addition, if reference techniques become accepted, it is important that species of known behavior be available in lyophilized form from type culture collections. The following additional criteria were proposed for suitable reference strains.

1. As far as possible, they should be suitable for testing several agents.
2. They should be similar in growth rate, nutritional requirements, and temperature optimum to rapidly or moderately growing pathogens.
3. They should be fully defined both as regarded their microbiological characteristics and their susceptibility to a wide range of antibiotics.

4. Strains should be homogeneous and genetically stable even after repeated subculture (28).

Representative strains of microorganism useful for many purposes have been deposited in the American Type Culture Collection (ATCC) and other standard repositories. A number of these are in widespread commercial use in screening for antibiotics. Activity against these particular microorganisms is likely to be meaningful because of extensive antibiotic experience with them and our knowledge of the specific potency clinically useful antibiotics exert against these strains (56).

The stress in any antimicrobial susceptibility test should always be placed upon employing standardized condition; for routine testing, the environmental conditions should not be altered from those that are optimal for the growth of the microorganism in vitro. When the environmental conditions are optimal, the greatest variation in an antimicrobial susceptibility test is introduced by the inoculum density. Any difference in the susceptibility of strains of the same bacterial species to a given antimicrobial agent, except with species that produce antibiotic - destroying enzymes such as penicillinase, are a reflection of the homogeneity or lack of it in the microbial population. An inoculum, therefore, that does not represent a good cross section of the population can lead to erroneous results (8).

Any attempt to screen large numbers of plant samples, whether from a chemical or biological point of view, involve inherent problems that must be taken into consideration when the results are interpreted The possibility of collecting plants which have variable chemical

composition due to ecological factors, varying collection times or due to the existence of chemical races of plants is real. Also, it is quite possible that separate plant organs, i.e. fruit, root, leaf, etc., each could give a distinctly different biological activity when compared with the effects of whole plant extracts (30).

In testing a given plant it was found desirable to use portions of all available parts, e.g. roots, stem, leaf and reproductive organs. In certain cases, the inhibitory substance was found to be produced by an enzyme acting on an inactive procursor, the enzyme being located in one part of the plant, the procursor in another; unless both parts were used together in the test no inhibitor was produced (63).

In selecting a method for testing for bacterial inhibition a number of factors have to be borne in mind. The method must be relatively simple and should require a small amount of material, since only small amounts of some of the plants to be tested are available. These two considerations alone are felt to be heavily in favour of diffusion method as compared to dilution methods.

However, the drawbacks of the diffusion method are :

1. it is not quite as sensitive as the dilution test,
2. inhibitors which will not diffuse through agar will give a negative result.

In view of the first objection, it is not surprising that certain materials which have been found by other workers to have a strong bactericidal action have not been given positive results (63).

It was decided to limit pre-diffusion to 30 minutes because this appeared to be optimum for reproducibility and practicality. There are theoretical advantages to a longer pre-incubation period, but there are probably offset by the problems of room temperature variation at different times of the year (28).

It was also decided to define agar depth precisely even though its effect on zone size has been shown to be remarkably small. This was done because there was a measurable influence of agar with some highly diffusible agents, and because of the risk of interaction with other variables (28).

The rate of diffusion, as well as the deterioration or inactivation rate, is far from identical for different chemotherapeutics. Therefore, when discs containing the same amount of different agents are placed on the surface of a semisolid medium, the joint influence of these two factors makes the concentrations of active drug after the same time and at the same distance from the discs very different (91).

It is evident that the size of the ring of inhibition of growth depends primarily not only on the antibacterial activity but also on the rate of diffusion of the antibacterial through the agar (88).

There is clearly no 'right' medium, and decisions as to selection of a routine medium must be based on considerations of convenience, cost, and reproducibility. For a reference formula, reproducibility is paramount (28).

A major source of variation is subjectivity in reading results, and this has usually been enhanced by inadequate definitions of end-point criteria. We have strictly followed descriptions of end-point to reduce the extent of this variable, but have not eliminated it.

In our study four solvents are used successively according to their polarities from the lowest to the highest, i.e. we started with petroleum ether, followed by ethyl ether, ethanol and water. With ethyl ether as a solvent many of the plants were found to contain some type of antibiotic substance. On several occasions, all the plants in a collection would have inhibitory substances in this extract. Ethereal extraction will remove chlorophyll, waxes, and sterols. It will also denature proteins and enzymes. The use of ethyl ether as a solvent bring up the question whether the dissolved chlorophyll acts as the inhibitory agent. This has not proved to be the case, as many plants tested have shown no activity from the ethereal solutions containing chlorophyll. Adsorption experiments by Carlson and Douglas (1948) with charcoal and kaolin have shown that chlorophyll was not the active agent (18).

The solvents were selected to yield extracts of varied types of material in which potential inhibitory substances might exist. An antibiotic which can only be isolated from fresh juices is likely to be very unstable and this process is very exacting in its time requirements, not lending itself readily to routine operation in temperate climates (56).

Effects of solvents control used, e.g. petroleum ether, ethyl ether, ethanol, water and effect of blank-disc showed no activities against microorganisms. Hence whatever results yielded in our experiments are directly influenced by plant extracts.

The results from Table 7 agree with our purpose of study that most of the medicinal plants tested should, more or less, show inhibitory properties against eight representative microorganisms which are suspected to be causative agents of many diseases.

A negative result does not necessarily prove absence of such substances in the plant concerned, for it is recognized that method of preparation may not reveal all antibacterial substances. Also the possibility of enzymic destruction should be borne in mind.

In addition to the inhibitory effect of some plant extracts a peculiar phenomenon of disturbed growth and very often of definite stimulation of the test microorganisms was observed. These phenomena were similar to those described by Abraham et al. (1941). They appeared as halos of varying sizes surrounding the zones of inhibition. The stimulation was in some cases of extraordinary strength (1).

It can be seen from paper of Lucas and Lewis (1944) that stimulative principles may be present in plant tissues together with inhibitors. A simultaneous action in this sense might explain the observation mentioned. It is also regarded possible that the inhibitor as it penetrates the agar becomes diluted to such a degree that its action reverses (49).

Lucas and Lewis (1944) noted that certain plants tested by other workers using different procedures do not give a positive response in the plate tested. It is believed that the failure of some plant extracts to respond positively to the plate test is due to the low degree of their diffusibility (49).

It should be noted that many volatile oils possess antiseptic properties; the antibacterial, antimicrobial, and antifungal activities of essential oils and perfume oils have been the subject of a series of investigations. This bacteriostatic effect may have been responsible for the high value placed upon certain spices of the Babylonian period (24).

Cavallito and Bailey (1944) summarized that a new type of antibacterial has been isolated from the cloves of Allium sativum Linn. The product, which has been named allicin, is a colorless oil, approximately 2.5% soluble in water, and relatively unstable. The antibacterial action has been demonstrated against both Gram positive and Gram negative bacterial (20).

Lutomski et al. (1974) showed that alcohol extract, curcumin and essential oil from Curcuma longa Linn. rhizome restrained growth of most microorganisms. An alcohol extract and essential oil showed bactericidal activity whereas Curcumin reacted as bacteriostatic agent with respect to Staphylococci (51).

The values of MIC of medicinal plants from Table 8 are according to our test conditions as described in previous chapter and cannot be compared with other values of MIC whose test conditions are varied.

As is seen from Table 8 many plants showed high activities, however, the values of MIC are higher than those of antibiotic control under the same test conditions because they are still crude extracts.

Representative microorganisms tested are susceptible to a wide range of antibiotic controls as can be seen from Table 9. There are very few antibiotics which can inhibit growth of Lactobacillus fermentum (Table 3). This fact can also be applied to our medicinal plants studied (Table 8).

Most active antibacterial extracts showed negative to chemical tests (Table 10) because

1. we only test for the presence of active substances in alkaloid and glycoside groups, but actually the active substances may belong to other groups aside from those mentioned.
2. the stability of the active substances during the wilting and drying of the plant also varies greatly.
3. active substances may degrade due to heat and the method used.
4. the amount of the chemical constituents in the extract may not reach the minimal sensitiveness of the particular test.

Groups of substance which we did not test are, for example, coumarin, tannin, cyanogenetic glycoside, etc. Coumarins occur in all plant parts and are found widely spread in various plant families. They are found especially in the Gramineae, Orchidaceae, Leguminosae, Umbelliferae, Rutaceae, Labiatae, and others. Coumarin itself has low antibacterial activity, dicoumarol has shown excellent activity against

certain bacteria. The most important coumarin-type antibacterial agent is the antibiotic, novobiocin (82).

Percentage yields (Table 11) are based on dry basis and conditions used as described in previous chapter on materials. Percentage yields vary with moisture content, locality, time, and maturity of plants.

The specificity and potency of extracts of plants of one family tend to be similar throughout the family, (Table 12), e.g. Liliaceae, Zingiberaceae, Caesalpiniaceae, suggesting that similar types of antibacterial substances occur in those species of the family. Inhibitory substances are in some case distributed throughout the plant, in others restricted to one part (63).

From Table 13, the most sensitive microorganism in their successive order against medicinal plants tested are Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa, Streptococcus faecalis, and Lactobacillus fermentum.

Ethereal extract has the highest effective ratio and water extract has the lowest one as shown in Table 14.

The distribution of the active substances in the plant varies in different cases, in many, e.g. Alpinia sp., it would seem to be contained in all parts of the plant; in others, the concentration in one part of the plant, e.g. in the cloves of Allium sativum Linn., greatly exceeds that in any other part (Table 15). Part of plant which is most effective in this study is leaf and it is not surprising

that leaves are often used in local remedies.

Another point to consider in the interpretation of data presented herein is the possibility of additive, synergistic or antagonistic effects due to the heterogeneous character of phytoconstituents in the extracts evaluated. Only the latter of these effects would present problems as far as primary detection of activity in plant extracts is concerned. It should be pointed out th at there is a distinct possibility of evaluating an extract with a specific type of useful and interesting biological activity, wherein this activity was not apparent due to the other substance(s) which exerted toxic effect. At this time, no reasonable approach to the solution of such a situation, other than a complete phytochemical investigation of all "toxic" extracts, is to be suggested (30).