PHARMACOGNOSTIC STUDY ON THE DIFFERENT SPECIES OF *Dioscorea* AVAILABLE IN DARJEELING AND SIKKIM HIMALAYAS WITH SPECIAL EMPHASIS ON THE PRODUCTIVITY OF THEIR DIOSGENIN CONTENT

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BY

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This is to certify that research work embodied in the thesis "Pharmacognostic study in the different species of *Dioscorea* available in Darjeeling and Sikkim Himalayas with special emphasis on the productivity of their diosgenin content" has been carried out by Miss Bhanu Gautam, M. Sc., B.Ed. under my guidance.

She has fulfilled the requirements relating to the nature and period of research. It is also certified that the thesis in corporates the results of original investigation made by Miss Gautam in Pharmacognosy Research Laboratory, Department of Botany, North Bengal University under my guidance and supervision and the thesis now submitted for the fulfilment of her Ph.D. degree in Science (Botanay) under the University of North Bengal has not been submitted previously for any degree whatsoever.

Dated : 19.3.2001

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In Proceedings of the National Seminar on Plant Biodiversity - Systematics, Conservation and Ethnobotany (in press)

2. Abstract

Applicaiton of a newly established colorimetric method for quantitative estimation of diosgenin in some wild species of *Dioscorea* available in Kalimpong, West Bengal. P. K. Basu and B. Goutam

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ABBREVIATIONS

aq	:	aqueous
&	:	and
BA	:	Benzyladenine.
BAW	:	Butanol : Acetic Acid : Water
BAP	:	Benzyl amino purine
b.p.	:	boiling point
C°	:	Centigrade
Cu	:	Copper
cm	:	Centimetre
CM^{-1}	:	per centimetre
Conec	:	Concentrated
D.C.O.M.P.	:	Directorate of Cinchona and Other Medicinal Plants.
Distd.	:	Distilled
2,4-D	:	2, 4-Dichloro phenoxy acetic acid.
E.Longitude	:	Eastern Longitude
Fe	:	Iron
Fig.	:	Figure
g	:	Gramme
HCl	:	Hydrochloric acid
H_2SO_4	:	Sulphuric acid
hrs.	:	hours
HPLC	:	High Performance Liquid Chromatography
IAA	:	Indole Acetic Acid
IR	:	Infrared
lb.	:	Pound
kg	:	Kilogramme.
K	:	Potassium
λ	:	Lambda
l	:	Litre
M	:	Molar
max	:	maximum
mg.	:	milligramme
min	:	minute

M.S.	:	Murashie Skoog.
Mn	:	Manganese
ml	:	mililitre
m.p.	:	melting point
mm	:	millimeter
m	:	meter
(N)	:	Normal
N latitude	:	Northern latitude
NAA	:	Napthalene acetic acid
nm.	:	nano meter
n-BuOH	:	Normal Butanol
N-NW	:	North-North West
O.D.	:	Optical Density
O-phosphoric	:	Ortho phosphoric
p	:	Phosphorus
%	:	percent
P.C.	:	Paper chromatography
pet. ether	:	Petroleum ether
ppm	:	parts per million
P.D.A.	:	Ptato Dextrose Sugar
pH	:	Hydrogen ion concentration.
R.H.	:	Relative Humidity
Rf.	:	Relative flow
r.p.m.	:	rotation per minute
S-SE	:	South-South East
sq.cm.	:	square centimeter
sp.	:	species.
T.L.C.	:	Thinlayer Chromatography
UV.	:	Ultraviolet
V/V.	:	Volume/Volume
wt.	:	Weight
Zn	:	Zinc

GENRAL INTRODUCTION

The region of Darjeeling and Sikkim Himalayas is a part of the great chain of the Himalayan Mountains which stretches from the North Western to North Eastern boundaries of the Indian Union. It lies between 26°31' and 28°N latitude and 89°E longitude and occupies a portion where different great areas like China, India, Tibet, Nepal and Bhutan are very close together. After independence and the birth of Indian Republic the hills of Darjeeling was converted to Darjeeling district of the state of West Bengal and Sikkim was merged wtih the Indian Union as the 22nd state in the year 1975.

The characters of vegetation in the region is influenced by the strong laden monsoon wind from the South. The ramifying outer spurs have a heavy rainfall and are densely clad by moist forest of tropical and sub-tropical genera. The central portions of the gorges and valleys have a lesser rainfall and tend to bear a drier type of forest. During the last few decades Darjeeling and Sikkim Himalayas have been subjected to drastic deforestation like all other parts of the country. As a result, the soil of the region has been affected tremendously to cause overinsolation, leaching of mineral and soil erosion. In general, soil of the region is acidic in nature and very poor so far as its fertility status is concerned. It is thus necessary to survey the nature of the soil in relation to distribution of various species of plants including *Diascorea sp.* which are still existing after being adopted to drastic ecological conditions. Moreover, due to rapid growth of human population in the area, the growth of different plant species is being antagonised. It is for these reasons the ecological study in the region with special emphasis on soil conditions, drainage, geographical formation, rainfall, temperature, topography, physiology, human population, vegetation and land use have been felt desirable.

The prehistoric men and women as they advanced towards civilization in the lap of time became dependent on plants. Like the wild animals living in the forests, human being too in the day of yore used plants intuitively for food, shelter and even curing their many a malady and thereby kept their health in perfect state of fitness and lived a long life unlike the human folk of the present day trouble ridden world.

Such is the situation with the region of Darjeeling and Sikkim Himalayas. Many folklore are also known to the hill tribes about the miraculous cure and are being cultured by herbalists, medicine men, Lepchas and Lamas living more or less a secluded life in the region.

Sir J.D.Hooker the world renouned explorer of Darjeeling and Sikkim Himalayas however seemed not to have laid much stress on the healing properties of the indegenous medicinal plants in his monumental work namely, "Himalayan Journal" or "Notes on a Naturalist in Bengal, Sikkim and Nepal Himalayas", although he mentioned in glorious terms about the Lepcha's wonderful knowledge of plants in the region. Before the advent of the western medicines and their supply reached the hill people - the hill tribes for centureis mainly depended upon the indigenous plants for curing their diseases.

Gradual introduction of European system of treatment of diseases and discovery of synthetic drugs and antibiotics and with the advancement of Western medical science which are now reaching gradually even into the interior of the hill ranges, the primitive system of treatment of diseases practised in older times by the medicinemen and herbalists of the different tribes in this region is fast diasppearing. Moreover, due to inherent secretive nature of these herbalists and medicine-men the knowledge and use of some of the really efficaceous vegetable drugs are dying out with the old veterans and head man of the villages who are gradually superstitious and cherish on inherent belief that if the secrets handed down to them from generation to generation about the wonderful uses of plants and given out to unauthorised person, the efficacy of the plants will be reduced. It becomes therefore extremely difficult to extract authentic information from the hill folk on the use of the medicinal plants used in the treatment of various diseases by the hill men.

The last thirty years or so, witnessed a resurgence of interest in the traditional medicines and drugs all over the world. This is because of the fact that many undesirable side effects are seen in modern synthetic drugs, the cost of which again is very high now-a-days. Besides about 10-15 years of effort is required to develop clinically acceptible drugs. Moreover, it has

been felt that there is inadequacy of the modern drugs for the so called "Refractory diseases" like hepatities, amoebic dysentry, diabetes and degenerative disorders like rheumatoid pain and as immunomodulators. As a matter of fact plants are almost exclusive source of drugs for the majority of the world population. Even today Vietnam is almost exclusively depended on traditional medicines. China like India, has amulgammated traditional drugs with the modern medicines. Plant products constitute approximately 25% of all the prescribed medicines even in the most advanced countries like U.S.A. Japanese pharmacopoeia (1986) contains 123 drug plants both crude and pure active principles of which only 29 are used in Western medicine (Pakrashi, 1995; Dev, 1995). Very recently, Mangari et al (1998) have given much importance to conserve important threatned medicinal plant like *D. deltoidea* in India, Samanta and Das (2000) also supports the same situation of the plant in Darjeeling district, West Bengal.

The pioneering and most outstanding floristic contributions have come from Hooker (1855). Others were King and Pentling (1898) and Brushl (1926) who worked on orchids of Darjeeling and SikkimHimalayas. Besides the floristic works of Gammie (1894) and Hara (1966) are remarkable. But none of them gave any importance on the use of traditional or tribal medicines inthe region. In this connection the work of Biswas (1956) and Biswas and Chopra (1940) are worth mentioning because they have described 147 common medicinal plants in Darjeeling and Sikkim Himalayas with much emphasis on medicinal properties and various uses of the plants from ethnobotanical point of view.

Ethnobotany has gained importance now-a-days because of the fact that it is both a fascinating and rewarding subject. It includes the fudamental aspects of identification and cataloging of plants and plant products that are used by the traditional societies and tribal communities. Documentation of the fast disappearing of knowledge possessed by them, require a penchant for adventurous field work along with a knack for mixing with people and winning friends among the ones that are initially shy and non-communicative and who might consider the representatives of the urbanished world as aliens.

The aspects of qualitative evaluation of the use and management of the natural plant wealth the experimental assessment of the benefits derived from the plants and utilising the traditional ecological knowledge for biodiversity, conservation and community development have brought recognition to ethnobotaby as an important and crucial area of research.

Though much information about ethnobotanical research in other places in India is available but the report on this aspect especially in connection with Darjeeling and Sikkim Himalayas is meagre. Only a small fraction of ethnomedicinally important plants has been reported from this region so far (Tamang and Yanzone, 1982; Yonzone et al 1984, 1985, Basu, 1990). Very recently ethnomedicinal information of "Tura" a locally available *Dioscoea sp.* in Kyato (Nakamura 1998) has been pointed out but the proper scientific name of the plant has not been mentioned. Besides the ethnoveternary use of *D. bulbifera*and *D. pentaphylla* in Andhra Pradesh, India has also been reported but no specific role of the plants has been mentioned (Reddy et al, 1998). But recently Min et al (1998) has reported that yam of *D. opposita* has a very good role on Myocardial lecta-adrenoceptors of hypothyroid rabbits. In Japan, Haraguchi et al (1999) isolated a phenanthrene derivative to show antifungal agent in *D. delicata* which is generally considered as natural medinice in the region. Very recently Kelmanson *et. al.* (2000) has reported that extract of tuber of *D. sylvatica* and *D. dregane* are active against *Escherichia coli* and *Pseudomonas aeruginosa* respectively similarly Huetal (1999) perinted out that the ethanol extract of the yam of *D. composita* exibited bioactivity against *Pyriculariaoryzae.*

Thus it is very essential to study bioactivity of chemical constituent available in different ethnobotanically important species of *Dioscorea* distributed in the region of Darjeeling and Sikkim Hamalayas.

Proper identification of any medicinal plant or tribal medicine is very essential in connection with its purposeful utilisation. In this respect the knowledge of pharmacognosy should be utilised for the identification of medicinal plant.

It was not until 1815 that the term pharmacognosy was introduced by C.A.Scydler, a medical student in Halle, Germany. The name is termed out of two Greek words "pharmakon", the drug and "gnosis" - knowledge. The most comprehensive idea of the scope of pharmacognosy was presented by Fluckiger who stated that it was the simultaneous application of various scientific disciplines with the object of acquiring knowledge of drugs from every point of view (Tyler et al, 1976). In a restricted sense the definition of pharmacognosy implies a particular knowledge of methods of identification and evaluation of drugs.

As different *Dioscorea sp.* having ethnomedicinal importance, are identified by the local people only on the basis of morphological character, the knowledge of pharmacognosy involving organoleptic, morphological, anatomical and chemical evaluation of drug will be of much help in better understanding of even minor variation within the species. Very little information is available in connection with the pharmacognostic aspect of some common Indian Dioscoreas (Philip et al 1980). But during study, the authors considered different characteristic features of only the yam of the plant but not taking the consideration of leaf, stem and root of the species. The morphology of pollengrains of *D. prazeri* and *D. bulbifera* has been worked out by Samanta and Das (2000).

The detection of phytosterol has a very good role in connection with the identification of plant from chemotaxonomy point of view. Upto date much inportance has not been given in isolating phytosterol in *Dioscorea sp.*. Very recently Savikuri - Fodulovic et al (1998) have studied phytosterol in callus lines of wild *D. balcamea* in Yugoslovia.

Thus during investigation on ethnobotanically important species of *Dioscorea* in the region of Darjeeling and Sikkim Himalayas much emphasis has been given to study the plants from pharmacognosy point of view for botanical and chemical identification of different species of *Dioscorea.*

Immediately after the discovery of Marker et al (1943) that cortisone and other steroid hormones could be synthesised from diosgenin after being

converted to 16-DPA (16-Dehydropregenalone acetate) screening of plants was taken up in search of cheaper raw material for sapogenins.

After an intensive study by Correl et al (1955) it was concluded that species of *Dioscoera* were ideal for the source of diosgenin. DCOMP also started cultivation of *Dioscoera* in 1977 and by 1990 some 100 acres were under the plantation of *Dioscoera.* At the onset, the indegenous species of *D. prazeri* was tried but due to its high resinous principle, thecultivation of the crop was stopped. Two new species (*D. composita* and *D. floribunda*) were introduced in the region from Central America and commercial cultivation of these plants started for the first time in Darjeeling hills since 1980. These two are among the few *Dioscoera* speciee from about 50 Indian and over 600 trans world species that have been identified to contain diosgenin in amounts which are commercially feasible to extract. The DCOMP commissioned a Diosgenin factory during 1985-86 with an annualy installed capacity of 1500-2000 Kg. production of amorphous Diosgenin. Recently Dixit *et. al.* (2000) become successful to achieve genetic improvement of *D. floribunda* through recycling of clonal selection. Average diosgenin content in these clones has been found to be the highest.

In India the estimated requirement of diosgenin is around 100 tonnes peryear. It is estimated that due to emphasis on population control programme, the demand would go upto 150 tonnes during the next few years. The present production of diosgenin produced mostly from *Dioscoera deltoidea* was obtained from the forests of Jammu and Kashmir, Himachal Pradesh, UP and is approximately 20 tonnes annually. The raw material has been collected indiscriminately for thelast few decades and with this speed of exploitation without any definite programme of cultivation and conservation, the raw material is facing the risk of being exhausted within next few years. Because of the magnitude of the dmand and the exhorbitant prevailing cost involved, screeening of *Dioscoera* sp. in the ecological condition of Darjeeling and Sikkim Himalayas should be taken up and technolgy should be worked out so that productivity of *Diosgenin* could be increased in the yams of *Dioscoera* sp.

Biotechnology has slowly evolved during the early fifties and may be said to have come of age in agriculture only in the eighties. The pioneering researches include the discovery of phytochrome and its role in switching plant advances permits splicing and anncaling DNA sequences at will. Now-a-days, the definitions are usually oriented towards processes and industry related products. Thus in 1981 European Fedaration of Biotechnology defined it as "integrated use of biochemistry, microbiology and chemical engineering in order to achieve the technological application in the capacities of microbes and cultured tissues. In 1982, organisation for the Economic Cooperation and Development, defined biotechnology, as the "application of scientific and engineering principle to the processing of materials by biological agents to provide good and services".

Dr. T.B.Kenorey Jr. Administrator, Agricultural Research Service (USDA)1986, defined it as "those biological means used to develop process and products employing organisms or their components" (Dasgupta 1991).

Diosgenin is generally present in yams of *Dioscoera* in two forms of either free or bound. Free diosgenin is produced in low quantity as compared to that obntained after acid hydrolysis. But during acid hydrolysis for 6-7 hours much of the diosgenin content has been observed to be transformed to dicene form which is considered useless in steroidal hormone industry. Thus proper methodology should be achieved to minimise diene form of diosgenin. Diosgenin in fixed form always becomes linked with the membrane of the cell. During heat stressed condition of the yam of *Dioscoera* integrity of membrane structure is always lost thereby liberating diosgenin. Thus proper technology involving high temperature treatment of freshly collected yam of *Dioscoera* may be evolved in connection with the extraction of diosgenin avoiding cumbrous acid hydrolysis.

Methodology so far used for the estimation of diosgenin from plant are gravimetric (Selvraj 1971). Though estimation of diosgenin with the help of gas liquid chromatography (Tang et al 1978; Glyzine et al 1981), densitometric method using TLC scanner (Gunawan et al 1994) are available but these costly intruments are not available in all laboratories. Besides,

during gravimetric method it is very difficult to estimate diosgenin in small amount of plant sample. Thus a suitable method with the help of easily available colorimeter for quantitative determination of diosgenin is felt necessary.

With this background different species of *Dioscoera*, collected from the ecological condition of Darjeeling and Sikkim Himalayas have been studied from ethobotanical, chemical, biotechnological and pharmacogonosy point of view so that the information derived out of the work will be of much help in connection with purposeful utilisation of these plants in the region.

ABSTRACT

The geographical position of Darjeeling and Sikkim Himalayas lies between 26°31' and 28°N latitude and 89°E longitude.It occupies a partof the great chain of the Himalayas. Due to development of dense forests and unique vegetation, the region has long been recognised as a store house of various medicinal and aromatic plants including different species of *Dioscorea*. Since the discovery that diosgenin could be utilised as precursor for the synthesis of cortisome and steroidal hormones required for the preparation of contraceptive pills, a number of commercially important species of *Dioscorea* are being cultivated for the production of diosgenin. In order to meet high demand of diosgenin, the raw material has been collected indiscriminantly during the last few decades and with the speed of exploitation without definite programme of cultivation and conservation the raw material is facing the risk of being exhausted within the next few years and for this reason there is a need to search alternative source of diosgenin. During survey in the region of Darjeeling and Sikkim Himalayas it has been observed that different species of *Dioscorea* are being used by various ethnic population for different purposes. Altogether eight different species of *Dioscorea* such *D. alata* L, *D. kamoonensis*Kunth, *D. arachidna* Prain & Burkill, *D. sikkimensis*. Prain and burkill, *D. sativum* L and *D. prazeri* Prain & Burkill which are locally named as Ghartarul, Bhyagur, Bharlang, Niltarul, Bantarul, Sutuni, Githa and Cucurtarul respectively have been taken in to consideration.

With this background it has been the objective of the present work to study some chemical and botanical aspects of these species of *Dioscorea* for their purposeful utilisation in the region and for which the results and observations have been represented in seven chapters. Chapter I deals with the review of literature related to chemical, biochemical and botanical aspects of plants with special emphasis on *Dioscorea* sp., Chapter II deals with the ecological survey in Darjeeling and Sikkim Himalayas with special interest on altitudinal distribution of different species of *Dioscorea* at different places. Informations in connection with topography, physiography, drainage, geological formation, climate, human population, land use in the region have been collected. During survey different types of forests according to altitude and their vegetation have

been noted. Soil samples of twelve places at different altitudes have been examined to know different criteria i.e. pH, organic matter, average contents of K.P., Fe., Zn., Mn and Cu. It has been observed that percentage of organic matter increases with the increase of altitude. The available concentration of potassium and phosphorous has been estimated to become low to medium, Zinc and manganese are low in amount and others are normal. Available iron has been observed to increase with the increase of organic matter. Regarding distribution of the species, *D. alata* has been observed to be distributed from 0-9000 ft, *D. kamoonensis*, 3000-above 9000 ft, *D. arachidna* and *D. esculenta*, from 0-3000 ft, *D. sikkimensis and D. sativum,* from 3000-6000 ft but *D. arachidna* and *D. prazeri* from 0-6000 ft. Chapter III deals with the pharmacognostic study, of all the species to include botanical and chemical identification of all the species and materials and methods and results of which have been represented in two sections i.e. Section A includes observations on organoleptic, macrascopic and microscopic study of different parts of the plant. The report in connection with identification work on the species so far reported includes only the macroscopic and microscopic characters of the yam only. But in this part of work organoleptic study involving different criteria like shape, colour, markings, feeling to touch, taste and odour of leaf, stem and yam of different species, the macroscopic and microspic characters of root have been taken into consideration along with those of leaf stem and yams. On the basis of various characteristic features of different plant parts, a key has been prepared for quick identification of the species so that all the species which resemble so closely maybe identified with the help of low cost instruments. As some of the species of *Dioscorea* understudy are toxic to human health, key that has been worked out will be of much help to identify edible and medicinally inportant species. During the study on quantitative microscopy lowest value of stomatal index (19) and Pallisade ratio (30) have been observed in *D. prazeri* and *D. esculenta* respectively whereas the highest value for stomatal index (30) and pallisade ratio (59) have been observed in *D. sikkimensis,* and *D. sativum* respectively. Section B, includes results in connection with chemical investigation of the yam of different species of *Dioscorea*. During investivation, isolation, purification and characterisation of phytosterol and diosgenin have been taken into consideration. Following conventional method of phyto chemical analysis, dried powder of yams of

Dioscorea sp. have been subjected to soxhlet extraction with pertoleum ether for isolation and identification of sterols.The petroleum ether extract has been subjected to column chromatography for purification of phytosterol. For isolation of diosgenin, powdered yam has been made to paste after addition of water and subjected to acid hydrolysis followed by petroleum ether extractions. Stigmasterol has been observed to be present in four species of *D. alata, D. sikkimensis, D. bulbifera and D. sativum,* lanosterol in *D. kamoonensis* and sitosterol in *D. esculenta* and *D. prazeri.* It is very intersting to note that cholesterol is present in all the species including *D. arachidna.* As regards diosgenin *D. alata* and *D. esculenta* have been observed to contain 0.07 and 0.06 percent in the yam on dry wt. basis respectively. The percentage yield of diosgenin in other species are *D. kamoonensis* (0.50%). *D.sikkimensis* (0.60%), *D. arachidna* (0.70%), *D. bulbifera* (0.75%), *D. sativum* (1.35%) and *D. prazeri* (2.30%).

The isolated products have been identified after studying comparative behaviour of isolated natural product with the authentic sample in relation to m.p., mmp, P.C., T.L.C., and IR spectrum and chemical tests.

Chapter IV deals wtih the establishment of a new and rapid colorimetric method for quantitative determination of diosgenin in the yam of different species of *Dioscorea.* Most of the authors has so far been utilised costly instruments like GLC, TLC, Scanners etc. in connection with quantitative estimation of diosgenin. Sometimes gravimetric method has been used but it is not suitable for quantitative estimation of the same on microscale from a large number of samples generally occur during investigation on the different species of *Dioscorea.* Besides the value obtained from the sample of small amount inconnection with gravimetric estimation of diosgenin, does not show much accurate result. Moreover, purification of diosgenin has not been adopted in the methodology reported earlier. Thus a colorimetric method has been standardised on the basis of a chemical test for diosgenin after purification in presence of sulphuric acid and rosorcinol. The reaction mixture consists of 1 ml of diosgenin, dissolved in glacial acetic acid mixed with 1 ml of resorcinol (10,000 ppm) and 0.2 ml of conc. H_2SO_4. The absorption maxima of the colour of the solution has been worked out to be 510 nm. Diosgenin solution having the concentration

from 100 to 10,000 ppm has been observed to obey Becr's law. The proposed method is claimed to be a new one as the application of conc. Sulphuric acid and resoreinol has not been done earlier. Moreover, it is considered as a rapid one because it takes a very small duration of time for estimation of diosgenin in large number of samples. The colour becomes stable after standing the reaction mixture for 25 minutes and becomes stable for 40-50 minutes. The proposed method is supposed to be advantageous because of the fact that diosgenin can be determined from a solution having very low concentration. Only a few milligramme of dried plant material is sufficient for estimation of diosgenin. With the help of this method free diasgenin in the yam of different species of *Diascorea* have been estimated. The yam of *D. alata* and *D. esculenta* which are being used as food by the local people do not show any presence of free diosgenin as compared to others. The free diosgenin content in others species are *D. kamoonensis* (0.20%), *D. arachidna (0.30%), D. sikkimensis (0.25%), D. bulbifera (0.06%), D. sativum (0.40%) and D. prazeri (0.60%).* After separation of free diosgenin the respective sample of yam of each species of *Dioscorea* has been subjected to acid hydrolysis treatment to estimate bound form of diasgenin in *D. alata* (0.07%), *D. kamoonensis* (0.28%), *D. arachidna* (0.35%), *D. sikkimensis* (0.35%), *D. bulbifera* (0.74%), *D. esculenta (0.06%), D. sativum* (1.00%) *and D. prazeri* (1.60%).

Chapter V deals with the isolation and characterisation of antifungal constituent in the yam of cultivated species of *D. alata*. While workings with the isolation of chemical constituent in the yam of different species of *Dioscorea*, the crude natural product isolated from water soluble part of yam of *D. alata* and kept for a long time in laboratory condition no contamination of microorganism has been observed as compared to other isolated products kept under the same condition but having with dense fungalmat. The fungus maintained in P.D.A. culture medium has been identified as Aspergillus niger.

In order to identity the chemical nature of the inhibitor present in the water extract of the yam of *D. alata*, phytochemical analysis has been performed. The pure crystals (mp 287°C) isolated from methanol chloroform mixture, has been identified as saponin due to its characteristic frothing while boiling in water. It has been identified as Dioscin on the basis of super impossible IR spectrum

having characteristic peaks λ max 3350 (Broad) 1640, 1375, 1175, 1050, 850, 820, 720 cm^{-1} of the isolated product with those of authentic sample. Besides, their chromatographic behaviour has been observed to be the same. That the isolated product is dioscin has been further confirmed after identification of the aglycone part as diosgenin on the basis of of the same m.p. IR spectrum and chromatographic behaviour as observed in the isolated product and authentic sample. The sugar part has been confirmed as Rhamnose and glucose on the basis of comparing the chromatographic behaviour of the isolated sugars with the authentic ones. Chapter VI deals with the different ethnic use of the yam of *Dioscorea*. For a long time the yam of *Dioscorea* has attracted the attention of all people as a source of diosgenin and for this reason the consideration of other use of *Dioscorea* has been neglected. During survey it has been observed that different species of *Dioscorea* are still being used by different tribal communities as ethnomedicine and other purposes. All the eight species of *Dioscorea* have been taken into consideration and intensive field work has been done among 15 different types of tribal populations available in 22 different villages in Darjeeling and Sikkim Himalayas. Out of eight different species of *Dioscorea* the yam of six species such as *D. alata, D. kamoonensis, D. sikkimensis, D. bulbifera, D. esculenta and D. sativum* have been observed to be edible but *D. arachidna and D. prazeri* are non edible. In connection with use of yam of *D. esculenta* it has been observed that though local people eat yams but the cultivation of the species is restricted only to the Lepchas. Ethno religious use by the Nepali people has been observed to be restricted mainly to *D. alata, D. sikkimensis D. bulbifera and D. esculenta* on the day of "Makar Sankranti" in the month of January in each year. Ethnospiritual use of *D. arachidna* by Limbus and Rais peole has been observed to be conducted as per recommendation of Phedangmas and Bizuwas commonly named for the doctor of Limbus and Rais people respectively. As regards ethnomedicinal use of *Dioscorea, D. sativum, D. prazeri and D. bulbifera* are considered to be important. In connection with ethno vetarnary use of different species, yam of *D. kamoonensis* is being used as a remedy against "Bhyagute" disease of cattle, the scientific name of which is "Haemorrhagia septimae" caused by *Pasteurella boviseptica*. In modern therapy Sulphamezithin (5 gm/day) is generally used. Similarly yam of *D. sativum* is being used by the tribal people as a remedy against parasitic disease of cattle

caused by *Fasiola hepatica*. It is very interesting to note that whenever the yam of different species of *Dioscorea* is used as edible material either as food or as herbal medicine, the fresh yam is generally cut into pieces and boiled with water. The water extract is generally discarded and which is observed to be traditional method of use. But at time of use of yam against any infection on the external surface of human body the yam is generally crushed into a paste to apply externally as an ointment. In order to identify the biologically active natural product available in the water extract of the yam of *Dioscorea*, phytochemical investigation has been carried out and water soluble dioscin a rhamnogluside of diosgenin has been identified. On the basis of lytic property of "Rhamnoglucose", the chacotriose in dioscin, the saponin in the water extract of *D. alata* and other *Dioscorea* is being claimed as toxic factor in the edible species but having with antimicrobial activity in non edible ethnomedicinal species of *Dioscorea*. Chapter VII deals with the investigation in connection with the effect of high temperature on the yam of *Dioscorea prazeri* to increase production of free diosgenin. Normally the yam of the plant has 0.60% of free diosgenin out of total diosgenin content of 2.2% on dry wt basis. When the small species of fresh yams have been subjected to 45°C for 50 minutes the free diosgenin has been observed to increase maximum of 1.20% on dry weight basis. It is being claimed that glycosidase enzyme may be released during deterioration of tissue during high temperature treatment and becomes active to initiate hydrolysis of saponin, the glycoside of diosgenin to release free diosgenin accumulated in the tissue.

The enzyme has been extracted by acetate buffer prepared after adding 8.3 ml of 0.2 M acetic acid and was made upto 100 ml with 0.2 M sodium acetate solution to maintain pH at 4.0. The optimum time required for maximum production of free diosgenin has been worked out. It is being claimed that there is a possibility to utilise glycosidase to release diosgenin from the saponin i.e. glycoside of diosgenin replacing acid hydrolysis during which 30% of diosgenin is generally converted to pharmaceutically unimportant diene form of diosgenin.

So there is enough scope of isolation and purification of large scale glycosidase for its utilisation in enzyme technology for the production of diosgenin in pharmaceutical industry.

CHAPTER - I

REVIEW OF LITERATURE

Medicinal and Other Uses of Different Species of Dioscorea

Yams of *Diascorea alata* form a cheap source of carbohydrate food and are extensively used by the people of Assam, Bihar, West Bengal, Madhya Pradesh, Orissa and Deccan in India. They are of immense value during the period of scarcity of food. For use as food yams are washed either as a whole or in slices and cooked or baked to eliminate water soluble part of them. Even the best one among the cultivated yams cause irritation in the throat or a feeling of discomfort when eaten raw. The acridity is due to crystals of calcium oxalate.

The cultivated species is comparable to potatoe in taste and quality. The yams of *D. alata* are used for alcohol production. *D. alata* yams contain on an average 21% starch. The starch grains are transparent oval, rounded, or trangular in shape and do not separate easily during extraction with water. The yams are considered anthelmintic and useful in leprosy, piles and gonorrhoea (Kirtikar and Basu 1933; Chopra et al, 1956). The yams of *D. bulbifera* is also used mostly as a famine food. It is used for the preparation of starch in Japan. Poisnous alkaloids, volatile acids and calcium oxalate present in the yams should be eliminated by suitable treatment to obtain an edible product. The yams of the plant are used in Kashmir for washing wool and as fish bait. Dried and pounded tubers are used as an application for ulcers; they are also used in piles, dysentery and syphilis. Bulbils of wild species are used as an application for sores.

The yams of *D. glabra* are eaten in Andaman islands and Khasi hills, but are not much liked as they become gluey when cooked. The yams of *D. oppositifolia* are used as an external application, after grinding and heating, to reduce swellings (Kirtikar and Basu, 1933). The nutritive value of edible yams of *D. pentaphylla* is nearly the same as that of *D. alata*. The flowers are often collected and used as vegetable. Leaves are also eaten at the time of scarcity. Yams are used to disperse swellings and as tonic. The yams of *D. prazeri* contain

saponin and are used as fish poison and for killing lice. The yams of *D. puber* are edible and are reported to be good though it may emit an offensive odour when cooked. (Chopra *et. al,* (1956). Lee *et al* (1999) has established antidiabetic activity of *D. batatas.*

Importance of *Discorce* sp. in Connection with Production of Commercially Important Steroid Drugs

Production of steroid drugs may be accomplished in principle, using any of three different approaches; isolation of drugs itself from natural sources; partial synthesis of drugs from suitable precursors of plant origin and total synthesis of artificial drugs. The first two approaches are commonly used now-a-days for the production of drugs on industrial scale and the third one involves cumbrous form of synthesis in laboratory condition and which may sometimes become uneconomical in nature (Velluz et al. 1965).

Earlier steroid production relied on animal sources such as horse urine, bull testes and cow ovaries (Butenandt et al. 1934). Several tons of those animal products were required and it was menace to obtain them easily. As the demand grew for hormones, partial synthesis from cholesterol came into practice since 1933, and synthesis of hormones was put in the market (Fernholz, 1933; Butenandt et al., 1934; Chakravarti et al. 1960). But due to multiple chemical steps of synthesis of hormones involving huge expenditure the use of some what cheaper sterol such as stigmasterol was encouraged (Fieser and Fieser, 19659), but its steady supply was again limited.

The cost of progesterone obtained from plant sterol was observed to be around 80,000 pound per Kg. The increased amount of cost involvement and limited supply of starting material for the production of sex hormones encouraged further research. The artificial synthesis of sex hormones and cortisones involves a lengthy process and at the same time is an expensive one. For these reason naturally occurring steroids are, now-a-days, in great demand for their utilization during partial synthesis of cortisones and sex hormones (Applezweig, 1962, 1969; Djerassi, 1966, Tekada 1972). Contraceptive

hormones and anti inflammatory agents derived from corticosteroides are the two major groups of steroid presently manufactured on an industrial scale (Weston, 1976). The importance and widespread use of these steroid drugs has been discussed by Applezweig (1969, 1974).

The dramatic increase in the scale of antifertility agent in recent years necessitates a greater supply of naturally occurring steroid precursor from which these drugs are prepared.

Initially an attempt was made to isolate cortisone directly from the organ of animal origin. But the output was extremely insignificant. Only 0.5 gms. of cortisone could be isolated from 450 Kg of incised beef adrenal cortex (Chakrabarti et al., 1961). Gravity of this situation was realized from the fact that Schering Laboratory, Berlin, needed 625 Kg. of ovaries from 50,000 cows to obtain 20 mg. of pure crystalline progesterone (Butenandt and West Phal, 1934). Total synthesis of steroid involves cumbrous process and their commercial production requires huge expenses. So the conversion of cheap and easily available naturally occurring intermediates into desired steroid hormones appears to be the best way for their commercial utilization. This led to an intensive phytochemical survey on vegetable sources during the last three decades to search steroid precursor of plant origin which would be cheaper and potentially useful for the preparation of cortico-steroids, sex hormones and contractive steroids on industrial scale (Marker et al., 1977; Barua et al. 1953, COrrell et al., 1955; Chopra and Handa, 1963 ; Chakravarty et al., 1957; 1964).

During the last few decades, it has been noted that various steroid compounds such as stigmasterol (Fieser and Fieser, 1959), cholesterol (Fernholz, 1933; Butenandt et al., 1934; Chakravarti et al., 1956) sitosterol (Chawla, 1977), desozycholic acid (Sarett, 1946; 1948, Correl et al., 1955), sarmentogenin (Lardon and Reichstein, 1958), hecogenin (Aplezweig, 1962; Rule, 1975) were attempted to be utilized as the basic steroid precursor for the partial synthesis of desired steroid drugs. For this purpose different plant materials such as *Glycine max, Phytostigma venesosum, Strophanthus sarmentosus, Agave spp., Costus spp.*, and *Dioscorrea spp.* were being used by various workers.

Fig.I shows the molecular formulae of several compounds. From national point of view, the two starting materials which are of maximum interest at present in India are diosgenin and solasodine, a steroidal alkaloid.

In course of time diosgenin was noted to be more convenient in comparison to other precursors so far utilized for the preparation of sex hormones and contraceptive steroids and had been the choice of the world till the early seventies (Apple zweig, 1962; 1969; Dherassi; 1966; Tekada; 1972).

According to Hathi Committee's report (Chaturvedi and Sinha, 1980) annual requirement of diosgenin in India was estimated to be 60 tonnes and its annual production in our country was noted to be in the order of only 10-15 tones. The first synthesis of cortisone from dexoxycholic acid isolated from ox bile involved 32 successive steps (Chakravorty and Roy Choudhury, 1974). Cortisone and its derivatives are noted to be oxysteroids in nature whereas the sex hormones, including the oral contraceptives have no oxygen substitution in the molecule. Hecogenin therefore provides a partial starting material for the synthesis of the corficosferoids whereas diosgenin has been noted to be suitable for the manufacture of oral contraceptives and sex hormones. Diosgenin however can also be used for the corticosteroid synthesis by the introduction of oxygen into the 11, L-position of pregnene nucleus during microbial transformation.

The matter of research on steroidal chemicals was proved to be fruitful when Fuji and Malsukawa (1936) discovered diosgenin. Marker and his associate (1943) revealed the potential use of plant sapogenin for the synthesis of cortisones and other related drugs via 16-DPA. Since then diosgenin has been used as the most important and versatile precursor being capable of transformation to all the types of steroidal drugs. The demand for steroidal compounds has been increased considerably and some 600-700 tones of diosgenin are being used now-a-days annually with the world-wide use of hormones estimated to be 500 millions per annum (Panda, 1980). Strenuous efforts are being made to discover the high yielding strains of plants and to assure a regular supply of raw material by the cultivators of good quality plants and, in this respect, different species of *Dioscorea* play a remarkable role.

Tuber of many Dioscoreas, commonly known as yams have long been used for food as they are rich in starch. In addition to starch, some species contain steroidal saponins as well as other alkaloids. From suitable sources sapogenins are isolated by acid hydrolysis of the saponins. Preliminary fermentation of the material often gives a better yield (Chakraborty et al., 1958). The water insoluble sapogenin is then extracted with a suitable organic solvent.

Until 1970 diosgenin isolated from the Mexican yam was the sole sources for the manufacture of steroidal contraceptives (Bammi and Randhawa, 1975). With the nationalization of the Mexican industry, however prices increased to such an extent that manufacturers switched over to utilise different others precursor for the synthesis of steroid compounds.

Utilisation of Diosgenin for Production of Steroids in Phytochemical Industry

To the pharmacogonisist steroid chemistry has always been a fascinating subject. This is not only because of this complicated and interesting chemistry involved in steroid reactions but also because of the incipient recognition of numerous and diversified physiological functions and pharmacological effects, such as, influence on carbohydrate, protein fat and purine metabolism; on electrolyte and water balance, on the functional capacities of the cardiovascular system, the kidney, skeletal muscle, the nervous system and some organs and tissues.

Diosgenin is isolated from *Dioscorea* spp. by initial bydrolysis of the root with mineral acids followed by extraction of the liberated diosgenin in the hydrolized root cake by hydrocarbon solvents followed by isolation of the diosgenin from these solution. However, at least one company extracts the dioscin with polar solvents and isolate the diosgenin by hydrolysis of purified dioscin (Kunjithapadam, 1977). The outline of the scheme for the production of 16-DPA from diosgenin have been represented in Fig.2.

The 16-DPA is converted into the 16-17-epoxide with alkaline hydrogen peroxide. The epoxide is converted through the corresponding bromohydrin to

the 5-pregnene, 3-17 diol which is converted through a series of reactions of Reichstenin's Substances 5. Substances 5 is converted into hydrocortisone by a fungal hydroxylation and subsequently into prednisolone again by microbial enzymes. Recent work in this area includes use of immobilized enzymes of microbial cells for controlled transformation of this type. Fig. 3 shows the production of cortisone and Hydrocortisone from 16-DPA.

Progesterone is synthesized chemically but 11-hydroxylation of progesterone is caused by the fungus *Rhizopus arrizus*, later *R. nigricans* was found to hyxroxylate progesterone at 11-position in higher yields. 11-Hydroxy progesterone, available in high yield by microbiological oxidation of progesterone is also an attractive intermediate to cortisone (Dherassi, 1966). The outline of the synthesis of progesterone and 11-Hydroxy progesterone from 16-DPA has been represented in Fig. 4.

16-DPA is reacted with hydroxylamine hydrochloride and the resulting 20-Ketoxime is submitted to a Beckmann rearrangement to the amide which on hydrolysis with hydrogen chloride gives the 17-Keto compounds, DHA acetate (Fieser and Fieser, 1960).

DHA acetate is reduced to the corresponding 17-alcohol with sodium borohydride in mixed methanol tetrahydrofuran solution and the 5-androstene-3-17-diol-3 acetate is converted into testosterone in a seris of reaction.

Again, for the synthesis of methyl testosterone DHA acetate is reacted with methyl magnesium bromide to form methyl androstene diol which is oxidized by Oppenauer procedure to methyl testosterone. (Fig.5).

Commercially Important Species of Dioscorea in India and Abroad

As regards Dioscoreas, *D. composita*, *D. floribunda* and to lesser extent *D. spiculitora* and *D. mexicana* are used in Mexico and Guatemala commercially for production of diosgenin while in China, *D. singierensis* is the commercial source of diosgenin. Various commercially important species of *Diascorea* used in different countries in the world has been represented in Table - 1. In India

D. prezeri and *D. composita* are commercially used in eastern and north eastern parts of the country. Out of these species again *D. deltoidea* is being preferred much as it is very easy to obtain pure diosgenin from this source (Chaturvedi and Choudhuri, 1980). It has been noted by Sarin et al. (1974) that the supply of *Dioscorea* tubers obtained from wild resources are likely to be exhausted in next 10-15 years due to large scale collection but poor natural regeneration. The situation is being further aggravated due to indication of raising these plants as commercial crops without commendable success. Commercially important species of *Dioscorea* now-a-days utilised in pharmaceutical industry in India has been represented in Table - 2.

Table - 1: The Commercially important species of *Dioscorea* used in different countries in the world :

Sl. No.	Species	Name of the country
01.	*Dioscores bakanica*, Kosanin	Europe
02.	*D. belixensis* Lundell	Central America
03.	*D. composita* Hemal.	Mexico
04.	*D. deltoidea wall*	Nepal
05.	*D. floribunda* Mert & gall	Mexico, Central America
06.	*D. friendrichalli* Kounth	"
07.	*D. hundurenzis*, Knuth	"
08.	*D. medicana* Guill	"
09.	*D. spiculiflora*, Nemol.	"
10.	*D. sylvatica* Ecklen.	South Africa
11.	*D. villosa*, hundell	United States
12.	*D. singierensis* Kunth	China
13.	*D. prazeri* Prain & Burk	"

Table - 2 : Different species of *Dioscorea* yielding yams for commercial utilisation in India (Kunj thapada, 1982).

Sl. No.	Botanical Source	Sapogenin	% of Sapogenin	Habit and cycle
01.	*D.deltoidea* (Rhizone)	Diosgenin	2-5%	Growing wild in Himalayan region. Organised culture not very successful. Cycle : 5 years.
02.	*D.prazeri* (Rhizome)	Diosgenin	1-3%	North Eastern Region, can be cultivated in this region.Cycle : Not available.
03.	*D.floribunda* (Rhizome)	Diosgenin	2-5%	Central American sps grows well in peninsular and Northern India low altitude location. Cycle : 1-3 Yrs.
04.	*D.composita* (rhizome)	Diosgenin	2-4%	Central American sps. Cycle : Abour 3 Years

Cultivation of *Dioscorea sp.* in India

At a symposium held in Lucknow in the year 1952 Dr. R. N. Chakravarty of the School of Tropical Medicine Calcutta, was the first to suggest the possibility of exploitation of diosgenin containing Indian *Dioscorea sp.* He mentioned that *D. deltoidea* and *D. prazeri* growing wild in North Western and North Eastern Himalayan regions respectively contained appreciable amount of diosgenin. This

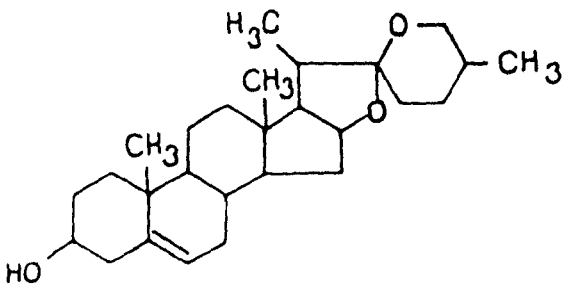
generated considerable interest in some pharmaceutical firms. An Indian company CIPLA entered the steroid field and began producing diosgenin in 1961. Subsequently CIBA-GEIGY, SEARLY and ORGANON also started manufacturing sex hormones. Glaxo (India) switched over to local diosgenin production and subsequently to its intermediate for producing beta-methasone. In 1966-67 CIMAP (CSIR) entered the commercial production of diosgenin in its drug factory, Jammu utilising the wild *D. deltoidea*. It was for the first time that in Eastern India the Directorate of Cinchona & other medicinal plants, Govt. of West Bengal started on a commercial scale for production of diosgenin and also entered to produce down stream products of diosgenin. At one time India depended almost entirely on the wild *D. deltoidea* for its diosgenin. the large scale collection of yams has resulted in dipletion of forest resources and in some areas complete eradication of the wild plant is well noticed. The natural regeneration of this species required more than seven years and it has been felt necessary to bring it under cultivation. Successful cultivation of *D. floribunda* and *D. composita* in Bangalore, Jammu, Goa and other parts of the country was reported from 1973 onwards.

Chemical Constituents isolated from different species of *Dioscorea* sp.

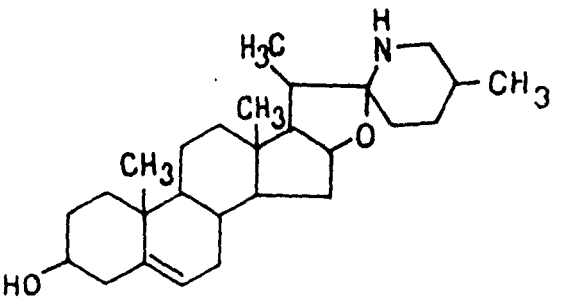
Different species of *Dioscorea* are the store house of various chemical constituents. Various chemical constituents which have been isolated so far from different species of *Dioscorea* are steroidal in nature and have been represented in the table 3.

Table- 3: Chemical constituents isolated from the yam of different species of *Dioscorea*

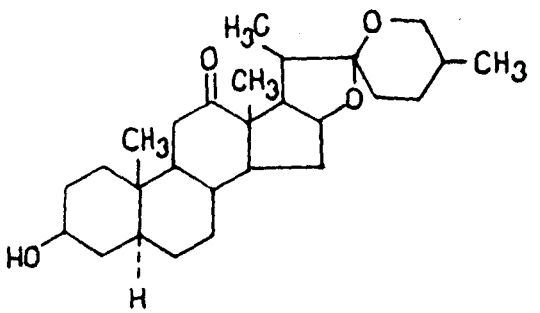
Species	Chemical Constituents	Reference
D. prazeri	*Diosgenin*	*Kunjitapadam, 1977*
	Prazerizenin A-glucopyranoside	Rajaraman and Rangaswami (1982).
	Prazerizenin A-ramnogluco pyranoside	Do
	Prazerizenin -D	Do
	Prazerol (9,10-dehydrophenathrene)	Biswas et al. (1988)
D. gracilima	Trisacharides of Diosgenin A and B	Rang & Vie Feng (1984)
D. panthiaca	Saponin	Li et at. (1986)
D. canposita	Diosgenin	Kunjithapadam (1977)
D. floribunda	Diosgenin	Do
D. deltoidea	Diosgenin	Do
D. hispida	Dioscorine	Lecte et al.(1988)
D. rotundata	Dihydrostilbene	Fagboun et at. (1987)
D. leullufera	p-hydroxyacetophenone	Gupta & Singh (1989)
D. septamoilea	Diosgenin B.	Lin and Yanyong (1985)
	Sitosterol	
	Palmitic acid	
	Diosgeninpalmitate	
	35-deoxytigogenin	
D. collettii var hypoglanca	Dioscin	Hu Dong et al (1996)
	Gracillin	Do
	Protoneodioscin	Do
	Protoneodioscin	Do
	Protogracillin	Do
	Methylprotodioscin	Do
	Methylproteodioscin	Do
	Methylprotogracillin	Do
D. delicata	Furostanol saponin	Haraguchi et al. (1999)



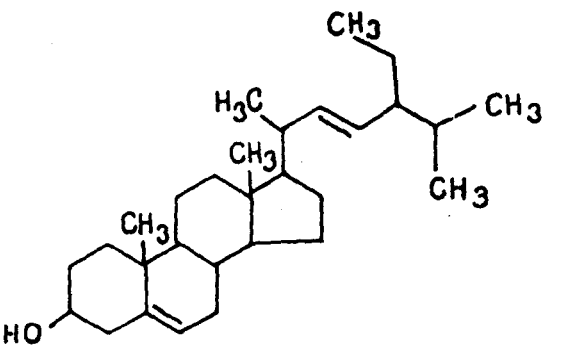
Diosgenin



Solasodine



Hecogenin



Stigmasterol

Fig: 1. Different types of Steroids and Steroidal alkaloid and their molecular structure utilised for the production of 16-DPA.

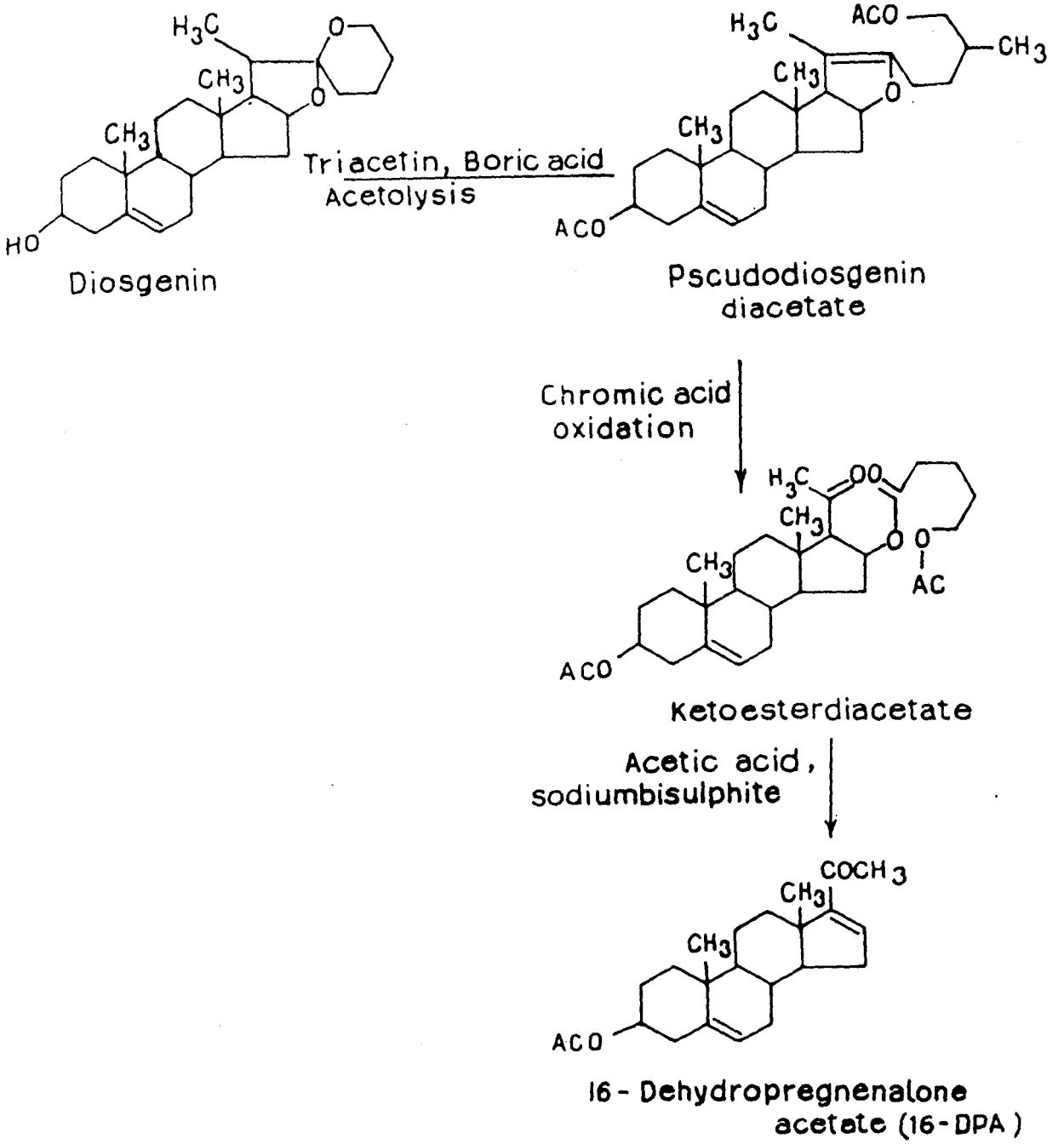


Fig. :2. The scheme representing production of 16-Dehydro pregnenalone acetate from Diosgenin .

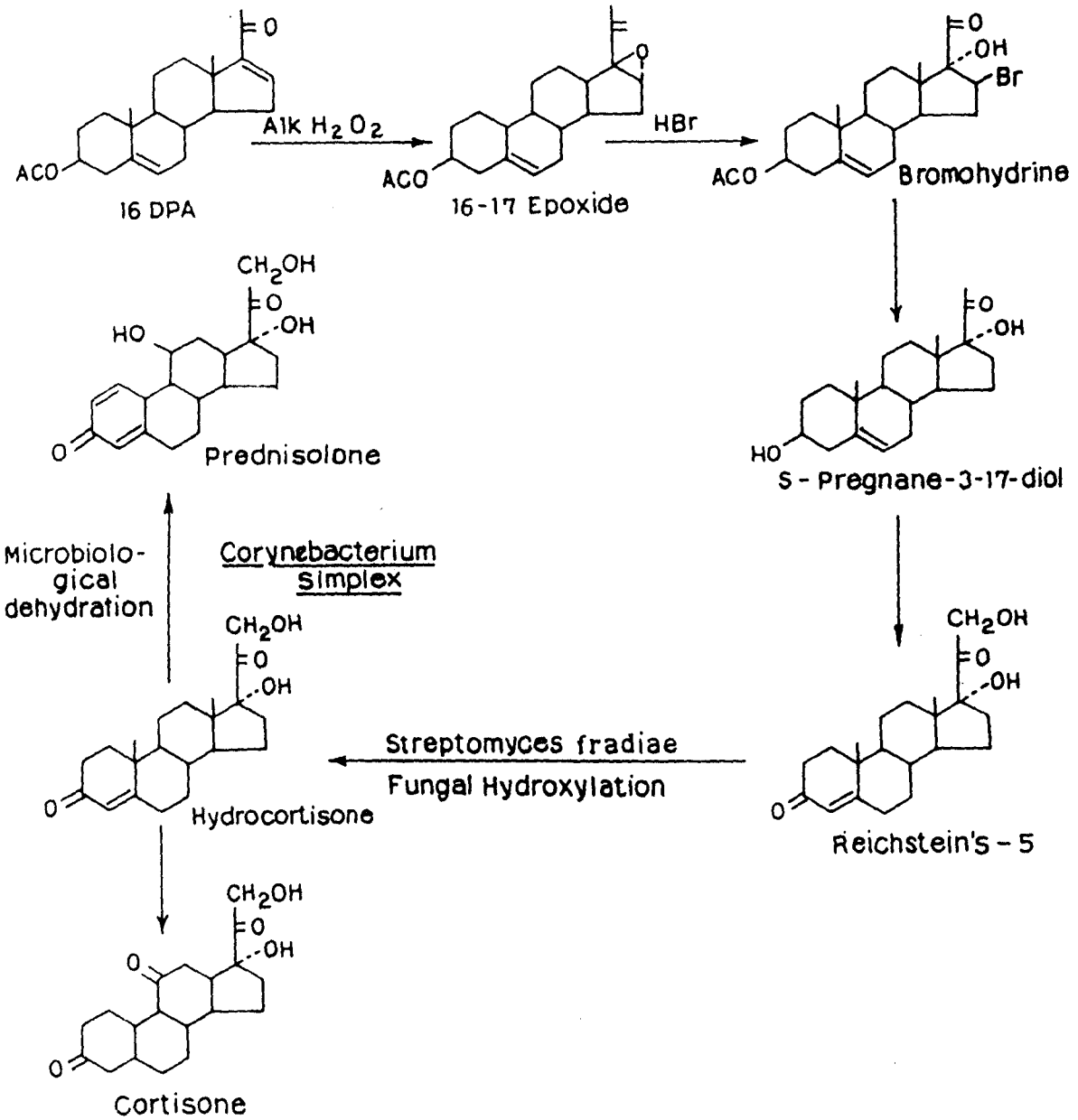


Fig: 3 Shows synthesis of corticosteroids from 16-DPA.

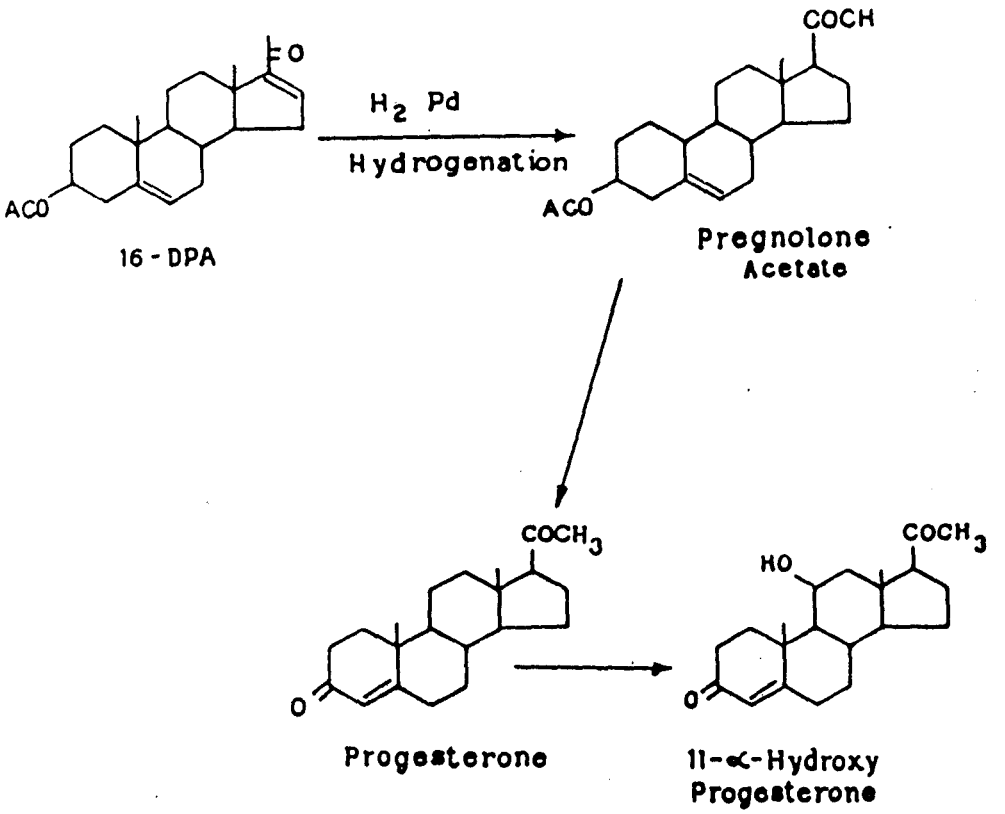


Fig. 4. Shows synthesis of progesterone and 11 - Hydroxyprogesterone from 16 - DPA.

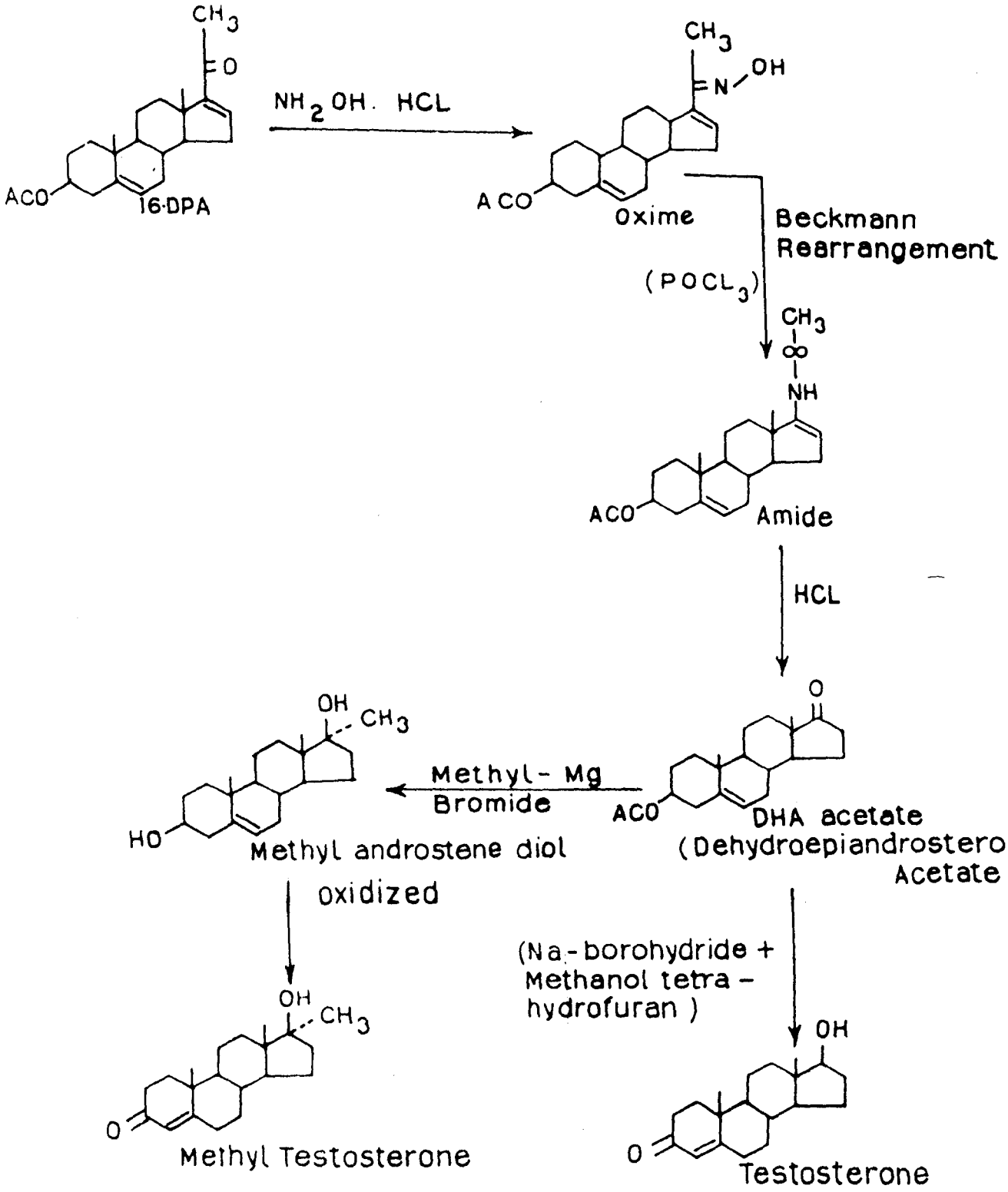


Fig. : 5. Shows synthesis of Testosterone and Methyl Testosterone from 16 - DPA .

Isolation and Purification of Diosgenin in *Dioscorea* sp.

The chemical method recommended by Marker et al, (1943) for the isolation of sapogenin have been noted not to be feasible from commercial point of view. According to their procedure the steroidal saponin extracted by ethyl-alcohol from ground plant material, was acid hydrolized to liberate the sapogenin from the glycoside. The crude sapogenin was obtained after ether extraction of the hydrolyzate and was purified by charcoal and recrystallized several times. Often recrystallization in the acetate form was required before pure sapogenin was obtained.

A procedure for isolating diosgenin, somewhat similar to the one described by Rothrock et al. (1957) has been presented in a recent patent by Sarin et al. (1976). The process for the isolation of diosgenin from tuber consists of three major operation of the tubers, hydrolysis of the saponin and extraction of the diosgenin. Rothrock et al. (1957) found that hydrolysis of fresh pulverised tubers with 2N HCl at boiling temperature for two hours was sufficient for hydrolysis of saponin. Chakraborty *et. al.* (1958) standardized a procedure in which formation of pharmaceutically in unimportant diene form of diosgenin (Fig. 6) Acid hydrolysis was avoided by aqueous hydrolysis and simultaneous extraction of sapogenin. Subsequently, Chakraborty et al. (1970) used a modified method in which they used 2N HCl in the ratio of 1 : 10 for hydrolysis of dry powder sample for 5 hours in a boiling water bath. Crude sapogenin obtained on extraction with pet. ether (40°-60°C) was washed with NaOH solution and evaporated to give a product which was chromatographed over neutral alumina. Ralatively pure sapogenin was then acetylated and diosgenin was estimated in the acetate form. Preston et al., (1961) used (1.5)N HCl to hydrolyze the sample for 5 hours. Selvaraj and Subhash Chandra (1980) observed that the hydrolysis of dry powder of *D. floribuna* with 2.5 (N) HCl for 2 hours would give a product which, on chromatography over alumina would offer diosgenin having the yield of 86.8-87.6% of total sapogenin. Chakravarti *et al.* (1961) proposed a procedure in which the oven dried material was powdered and transfered to a soxhlet extracter and extracted with light pet. ehter (40°-60°C) for 8 hours. The extract was concentrated to about 50 ml., when crystal of diosgenin began to appear. At this

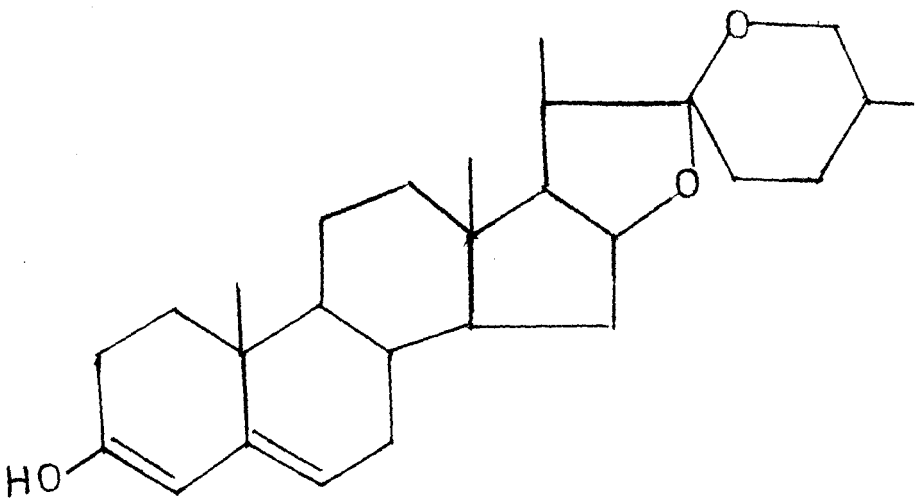


Fig.6 Diene form of diosgenin

stage the flux containing the crystal was refluxed for 1 hours. After cooling crystals were filtered through a sintered crucible and washing was performed with a fresh quantity of 50 ml. of cooled pet. ether to make the crystal free from any colouring matter. Further crystals, if any from the mother liquor were similarly recovered and added to the bulk. The crucible was dried in an oven at 100 degree centrigrade for 2 hours, dessicated and weighed.

Later Gandotra et al. (1977) suggested a suitable method which was modified later by Panda and Chatterjee (1980). According to the procedure fresh rhizome was cleaned with running warer. After cleaning the roots the excess of adhering water was removed by wipping with a clean cloth. The rhizome was dried, ground and hydrolyzed in an autocleve at the pressure of 15 lbs. for 15 minutes in presence of 4% HCl. The slury was filtered under reduced pressure. The residue was washed with distilled water to free it from acid. The acid free residue was dried in an oven and extracted with hexane in a soxhlet apparatus for 8 hours. The solvent extract was concentrated, chilled in ice to obtained diosgenin. The diosgenin was weighed after drying in an oven for 2 hours at 80°C.

It has been noted from the literature that during the preparation of tubers for processing, either fresh or dry tubers were pulverized through a micro pulverizer (Bantam) equipped with 0.013 inch (Slotwidth) herringbone screen (Rothrock et al. 1957). Moriss et al (1958) used sliced fresh tuber. These were macerated in presence of water with a high speed blender. According to Chakraborty et al. (1958) the size of the particle of tubers is of considerable importance from the stand point of efficient extraction. Sixty mesh powder found to be suitable for extraction.

It has also been noted that during hydrolysis of saponin different workers expressed different views. Earlier workers, Marker et al. (1942); Fuji and Mathsukuwa (1936); hydrolyzed under conditions varying from 2 N HCl refluxed for 2 hours to alcoholic H_2SO_4 treated for 20 hours. In none of these cases optimum hydrolysis condition was shown. Rothrock et al. (1957) found that diosgenin was completely hydrolyzed by 4 N HCl solution refluxed for 4 hours.

During extraction and purification of diosgenin from *Dioscorea sp.* various

hydrocarbon solvents have been used and out of which pet. ether, Skellysolve B, Skellysolve C, and Esso heptene proved most useful (Rothrock, 1957). The yield of diosgenin for assay and development work was based on crystalline product having m.p.200°C. This material of about 95 to 100% purity was found satisfactory in the usual test for the preparation of 7-dehydrodiosgenin in acetate. According to Rothrock (1957), in order to obtain diosgenin of still higher purity it can be prepared by recrystallization from methyl-ethyl-ketone, ethyl alcohol-acetic acid 1:1 or diosgenin after column chromatography over neutral alumina using chloroform acetone (3:1) as eluent. The extract was crystallized from methanol to give fine needle shaped crystal (m.p.202-204°C). The purity of the product was checked by TLC over silicagel G using benzene-chloroform (1 : 2) solvent system. The spot was detected under UV light after spraying with 50% phosphoric acid.

Quantative Estimation of Diosgenin in *Dioscorea* sp.

In connection with various methods so far utilized for the quantitative determination of diosgenin, Selvaraj (1971) extracted diosgenin and estimated it gravimetrically. He took yams of *Dioscorea* which were washed, dried and powdered. 20 gms of the powder was taken, hydrolysed with HCl (2.5 N) for 3 hrs. and filtered. The residue was repeatedly washed to make it acid free. It was then dried for 6 hrs. at a temperature of 100°C. It was later on extracted with petroleum ether (40°C-60°C) in soxhlet for 3 hrs. The extract was concentrated and cooled and then filtered. The diosgenin was calculated on dry weight basis.

Glyzine et al (1981) utilized GLC for quantitative determination of diosgenin in *Dioscorea.* The raw material was ground and hydrolysed with 2(N) HCl. The product obtained was dried and extracted with a mixture of chloroform and ethyl alcohol (1:9 v/v) to obtain diosgenin. After isolation GLC was used for estimation of diosgenin content. Later the GLC technique was developed by Azorkova *et al.* (1978). They determined the diosgenin content in *Dioscorea* to 0.11-2.71% using GLC. Tang et al (1979) also utilised the GLC for quantitative analysis and identification of steroidal sapogenins of *Dioscorea.*

Pasehnichenko et al (1978) estimated the colorimetric method for the determination of glycoside bound diosgenin inthe suspension culture of *D. deltoidea* using concentrated H_2SO_4 and 1% formaldehyde.

Recently Raman et al (1995) studied diosgenin involving ^{1}H and ^{13}C spectral assignment of the compound by using two dimensional NMR technique and resulted in unambignous spectral assignments specially in the convoluted region of the spectra.

Bio synthesis of Diosgenin and related steroids in *Dioscorea* sp.

It has been noted that steroidal saponin arises via the mevalonic acid pathway to produce "squalane". The subsequent cyclization of squalane to give cholesterol is well established (Croey et., 1966). Cholosterol has recently been shown to be incorporated into a number of C-27 sapogenins with side chain cleavage (Haftman, 1967). Consequently it appears that cholesterol is rapidly formed and metabolised. The ability of cholesterol to serve as precursor for other 27 carbon, sterols was shown by its conversion to tigogenin, gitogenin and diosgenin (Bennett and Heftman, 1965). Joly et al. (1969) showed that open chain saponins (5-Furostene 3, 22, 26-tropl 3.chaco side 26, D-glucopyrenoside) are formed from cholesterol. He showed that in *Dioscorea floribunda* homogenates choleserol was converted directly to dioscin i.e. diosgenin glucosides.

In plants the sapogenins are combined with sugar to form the saponins, Generally, the sugars are in a branched chain and are attached to the C-3 position of the steroid moiety (Joly et al. 1969 a,b).

According to some authors (Bennettal Haftman) 1965 ; Joly et al. 1969) cholesterol and sitosterol are the precursors to form of saponin and cholesterol is directly converted into diosgenin, Sitosterol, however, require a two carbon unit from C-24 and may proceed oxygenation. The sequence in which oxygen is introduced at position 16, 22 and 26 is unanswered, but indirect evidence strongly suggests that oxygenation at C-26 is the first step (Bennett et al. 1970) and is not dependent upon a 24 bond (Joly et al. 1969). Cholesterol, however does not

appear to be an obligatory step in the bio-synthesis, since desmosterol is converted to saponin without going through cholesterol (Tschesche et al. 1974).

Factors Affecting Growth and Development *Dioscorea* sp. and Production of Diosgenin

Karnic (1975) reported that *D. prazeri* and *D. deltoideal* from different locations in India yielded varying percentage of sapogenin content at various stages of growth. Sapogenin concentration increased with the age of the tuber. The optimum content of sapogenin was found when the plants were just shedding i.e. in dormant stage, which appear to be the best for commercial exploitation.

Gangadhara (1974) pointed out distinct effect of external and internal factors on productivity patterns of active principles in some *Dioscorea* sp. and such an increase in productivity was found to be functions of topographical, ecological, enviornmental as well as of some biochemical factors. Enyi (1970) found that there was a positive correlation between rainfall and vine growth, vine weight and tuber yield of *D. cayanensis* which required long growing season for maximum production; whereas in *D. alata, D. rotundifolia* and *D. esculenta,* much shorter growing season was required. According to Gooding (1970), rainfall below 100 cm during the eight months growing season appeared the limiting factor in *D. alata.* Wilson and Mapother (1970) concluded that in *D. deltoidea* plants raised from seedlings and set out in May, tuber yields were meximum after seventeen to nineteen months. Diosgenin percentage augmented during the first year after which there was a little fluctuation in its content.

Shelvaraj et at. (1971) investigated the distribution of diosgenin in one year old tubers of three sapogenin bearing species i.e. *D. composita, D. floribunda* and *D. deltoidea.* The intact tuber was separated into three different portions used generally for propagation i.e. crown, median and tip. The sapogenin content was found to be more in the dorsal portion in the tuber than the ventral portion in *D. composita* and *D. deltoidea* on fresh weight basis. However, in *D. floribunda* the ventral portion had the maximum content. Tip portion of the tuber in *D. composita,* the median portion of the tuber in *D. floribunda* and

D. deltoidea had the maximum sapogenin content. They also reported that the diosgenin content of *D. floribunda* tuber had closer correlation with the dry matter production of two years old tuber. It was claimed that *D. floribunda,* planted in April in Bangalore region, was the best as it gave quicker sprouts and higher tuber yields. Bammi and Randhawa (1975) did not find any correlation between girth of tuber and diosgenin content but a positive correlation was obtained between available phosphorus in soil and diosgenin content in tuber, whereas the level of available potassium in the soil was negatively correlated with diosgenin content.

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Bammi and Randhawa (1975) suggested that 40 to 60 gms. pieces of *D. floribunda* tuber as a planting materials were more economical. They also suggested that as the crown portion of the tuber contained less sapogenin compared to median and tip, the crown portions could be used as planting materials having the maximum survival percentage to get the maximum return. Nutritional conditions affect the general growth of the plants as well as actual

formation of active principles. Cruzado et al. (1965) claimed that complete fertilization in *D. composita* increased diosgenin content. The fertilization effect had also been studied by Ferguson and Hynes (1970) in *D. esculenta* and *D. alata*. Nandi and Chatterjee (1978) showed the phosphorus fertilization to enhance extension growth of *Dioscorea*. The enhancement of diosgenin content by phosphate and its decrease by nitrogen fertilizers in *D. bulbifera* var *pulchella, D. pentaphylla* and in *D. composita* have also been observed. Singh *et al.* (1981) observed significant interaction between N and P, N and K and N with P and K on diosgenin yield. They also noted that the tuber yield/plant and tuber production per unit area of *D. floribunda* to be increasing significantly with increase in N and P dose. Khan and Shakeel (1990) showed that application of N. P. fertilizer at 135 Kg N and 65 Kg. Phosphorus perhactre as basal does gave higher yield as compared to control.

After the discovery of photoperiodism in connection with the flowering of the plant Garner and Allard (1920) and various other authors have utilized the principle for commercial development of economically important plant in India and abroad(Hendricks et al., 1956 ; Wareing , 1996 ; Nitsch, 1957 ; Vence pure 1975 ; Nandi and Chatterjee 1978 ; Singh and Nanda, 1981). Akahori et al., (1970) reported that in *Dioscorea tokoro* this application of light increased the amount of yamogenin and tokarogenin in long day conditions. Akahori et al (1970) reported that the amount of sapogenin in aerial parts of *D. tokoro* could be increased by long day photoperiodic condition. Similar observation was also noticed by Karnick (1972) in *D. deltoidea*. Nandi and Chatterjee (1978) in *D. pentaphylla*, and *D. composita* and Wright et al (1989) in *D. Zingiberensis*

Light influences a number of biochemical, physiological and morphological characteristic of plants including steroid metabolism. Generally dark grown plants contain more steroidal constituents on dry weight basis than light grown plants (Duperon 1968; Bush et al, 1971). The bio synthesis of sterol from mevalonic acid has been observed to become higher (Bush and Grunwald, 1973). The light effect on metabolism gives rise to a change in the component of sterol very similar to that of senesence (Grunwald 1978). It is well known that light is one environmental factor that not only acts on photosynthesis but

also on the process of senesence. Wool house (1967), with ageing cell organelles, showed a loss in structure, when eventually only the plasmalemma and some empty residues remained and opined that reduced light intensities changed this process. The increased accumulation of some sterols in plants during this phase of plant growth has been linked to the disorganisation of intra cellular organelles (Duperon, 1971).

The effect of different quality of light responsible for growth and development of plant, opened a new avenue towards advancement in plant physiology. After that various informations are available in this respect, (Down, 1956; Moore , 1980; Walton et al., 1982; Rao et al., 1982). The effect of the quality of light has been considered to be due to the interference of phytochrome and in this respect information is available in connection with the structure and the function of the pigment (Parker *et al.*,1946; Borthwick *et al.*, 1952 ; Butler *et al.*, 1959; Siegelman and Butler, 1965 ; Hillmann, 1964; 1967). Phytochrome is a tetra pyrole structure and intermediate forms between red and far red of phytochrome exist.(Linschitz et al.,1966) These intermediates, represent different forms of the protein component of the phytochrome system although they represent two distinctive conformational states of the protein. This protein has in fact credited with enzyme activity (Tezuka and yamamoto, 1969) which raises the prospect for light regulation of metabolic processes. Very recently Woitzik and Mohr (1988) observed the control of gravitropism in plant by phytochrome and noted strong effect of red and far red light treatment on blue light mediated phototropism. Hunt et al., (1989) observed spectral quality of light influencing many aspects of plant growth and development. According to Karisson (1988). Phytochrome is not involved in the red light enhancement of the stomatal blue light response in plant. The stomatal response to blue light in plant was enhanced by back ground red light.

Lopez - Figueroa and Niell (1988) observed that the amount of Chlorophyll was accumulated in greater quantity in presence of blue light and pointed out the involvement of specific blue light photo recepter. Basu et al., (1988) observed that uptake of L-leucine could be enhanced by 50% over control by red light irradiation which was reversed by far red light. Reduction in loosening of cell

wall has been observed due to the effect of blue light (Cosgrove, 1988).

Work in Soviet Union Voskresenskaya, (1950) gave an early indication of light quality effects on photosynthetic products. Leaves were noted to be added more dry weight in red light than in blue light (Mc Cree, 1971), But in red light, 68% of the added dry matter was due to carbohydrates as compared to 42% in blue light Das and Raju (1965) has revealed that blue light stimulates accumulation of protein and other non - carbohydrate substances. Though diosgenin synthesis is very much related to carbohydrate metabolism (Benntt and Haftman, 1965 ; Jolly et al., 1969) but no report has so far been made in connection with the effect of quality of light on diosgenin But the effect at climatic factors on diosgenin production in *D. zingiberensis* has been worked out by Wright et al (1989).

Khan and Shakeel (1990) worked out the opitimum period of growth of D.deltoidea. They observed that monsoon planting provided more number of sprouts and higher yield of rhizomes.

From literature it appears that IAA retards the growth of plant and delays the onset of senescence of attached and detached leaves ; (Leopold, 1960) IAA is known to promote rooting in the stem cuttings of many plants (Thiamann and Behnke - Rogers, 1950). It has been supported by other workers; (Leopold, 1960 and Hess, 1962).

Although auxins have been shown to have variety of actions (Bonner and Varner, 1965) their effects on secondary products have been studied only sporadically.

It has been noted that auxins and phenoxy acids have significant effect on the production of steroids or other related compounds in both plants and in explant during culture; (Genus 1975, Hardman and Stevens, 1978; Joans). In their experiments IAA and 2, 4 - D have been noted to increase diosgenin content in several plants including *Dioscorea,* (Marshall and Staba, 1076).

Chaturvedi and Choudhuri (1980) reported that in tuber of *Dioscorea deltoidea*, IAA and 2, 4-D combination yieled maximum diosgein content.

Eversince the discovery of Kinetin as a stimulator of cell division in tobacco pith tissue (Miller et al., 1955) it has evoked considerable interest and is well known for its role in all phases of plant growth and development (Miller., 1961, Kuraishi, 1959; Steward et al., 1961). Seasonal changes in abscisic acid in *D. florilemda* has been worked out by Farooqui et al (1989) and seasonal variation in diosgenin content in this leaves of *D. composita* has been studied by Hensl et al (1987).

Bhatia (1978) observed the beneficial effect was of MH in connection with yield of certain secondary metabolites in plants. Bhattacharya and Varsha (1981) showed that MH inhibited rootings at higher concentration. According to Gupta (1970) MH delayed initiation of bolting, flowering and fruiting. He also noted complete inhibition of flowering at higher concentration of MH. Although MH may inhibit cell division (Greuaea and Atchison, 1950) it may promote cell enlargement also. MH did not inhibit internode elongation in plants but could produce early flowering in plant when applied during cold treatment (Moore, 1980).

Tatum and Curme (1951) suggested that the accumulation of sucrose in the leaves after treatment with MH might alter to the C/N ratio to the extent of causing collapse of pollen grains in plant. MH has been noted to affect diosgenin synthesis particularly when it has been applied at lower concentration (Mandal and Chatterjee, 1984).

As regards the effect of the chemical on carbohydrate metabolism it was noted that hydrolytic activities were high under the treatment of NAA. NAA did not appear to be related with root formation. NAA was noted to adversely affect synthesis of strarch. This might be of the reason of failure of NAA to promote root formation over control (Basu et. al., 1966).

NAA was observed to show male sterility in plants, (Moore, 1980). Production of seedless fruits, due to the treatment of NAA was noted by Gustafson (1936) and Hagemann (1937). NAA was found to be less deleterious to IAA and IBA (Boxy and Chatterjee 1967). Archarya Chowdhury (1968) showed that there was a rise of nucleie acid level as a result of NAA application.

He showed significant decrease of total nitrogen as well as the non reducing sugar. This is perhaps due to the fact that the conversion of sugar to nucleie acid might be facilitated by the exogenous application of NAA. IBA stimulated root growth in *D. floribuda* and *D. composita* cutting (Martin and Delphin, 1969).

Martin and Delphin (1969) noted that IBA stimulated root growth and treatment with ethylene chlorohydrine in a sealed container at the rate of 0.25 mg/gm air space was noted to stimulate root growth in *D. floribuda* and *D. composita* cutting. Dormancy in seed germination has been observed in *D. composita* (Viana and Felippe,1990)

Application of biotechnology in *Dioscorea*

Mass multiplication of Dioscorea through tisse culture was first demonstrated by Chaturvedi (1975).In a confirmatory report by Lakshmi Sita et al (1976), the role of tisse culture has also been emphasized in propagation of *D. floribunda*. Since the first publication (Chaturvedi, 1975), nuch advancements had been made by way of increasing this rate of multiplication, derivation of minimal rooting medium, standardization of techniques and testing.the method on a large scale. Clonal multiplication of *D. deltoidea* by in vitro culture of single node leaf cutting, and shoot apex culture was also reported for the first time by Chaturvedi et al (1977). Induction of rooting in stem cuttings was quite difficult as compared to similar explants of *D. florilenda*. In an optimum treatment containing 1.5 mg/l NAA + 1.0 mg/l indole butyric acid (IBA) or vice versa, only 50% cutting could be rooted. Later on it was found that a combination of 4 auxins i.e. NAA, IBA, 2-4-D and chlorogenic acid considerally improved the rooting percentage. A large number of invitro raised plants of *D. deltoidea* were observed growing normally in soil under glass house conditions.

Shoot apices of *D. deltoidea* measuring 1 mm in length and comprising meristam and 2 youngest leaf primardia were successfully regenerated in to complete plants withen a period or 90 days, which grew normally in potted soil and developed tubers (Chaturvedi *et al.* 1977) Besides clonal propagatan successful short apex culture is especially useful for the elimination of viruses,

several of which are reported to infeet Dioscorea (Water Worth *et al.* 1974).

There have been two reports on regeneration of plants from somatic callus cultures of *D. deltoidea* (Grewal *et al.* 1976; Mascarenhas *et al.* 1976), but the exact mode of differentation of plant lets was not brought out. Chaturvedi (1979) showed that the differentiating cells of callus cultures of *D. floribunda* and *D. deltoidea* followed the embryo genetic planted formation. By closely investigating the histology or anatomy of embryogenesis even the "single cell state" in embryoid formation was traced, in the proembryogenic tissue for both the *Dioscorea* spp. Embryogenesis was induced in the treatments containing 0.25 to 0.5 mg/l BAP plus 1 mg/l indoleacetic acid in both the calli of *D. floribunda* Embryogeneisis was augmented in tuber callus of *D. deltoidea* when the conc of IAA was increased to 3 mg/l in the above pair, where as in its leaf callus it could be induced, through to a lesser extent, by substituting Zeatin for BAP.

Chaturvedi (1979) isolated embryoids from the callus culture of *D. floribunda* Mature embryoids presented a large assay of different sizes and shapes from bell and funnel shaped to disc and star shaped, however the most interesting were the "dicotyledonous" embryoids.

In cultures showing optimum differentiation, even more than fifty plant lets were formed per culture. Further several "crops" of plantlets were obtained from a single differentiating culture by repeactedly excising the shoot and sub culturing the remaining portion. The callus regenerated plants showed variation in vigour, leaf, shape and size and in their ploidy level; from diploid to poly ploids and aneu ploids. Rarely, albino mutants to those having variegated leaves were also isolated from differentiating leaf callus of *D. floribunda* (Chaturvedi, 1979:)

An easy method for obtaining a large number of tetraploids of *D. floribunda* in a short time by the application of colchicine in aseptic culture was developed such tetraplord shoots were rapidly mass multiplied through in vitro culture of single node leaf cuttings and a large number of tetrapoid plants of *D. floribunda* were obtained growing in soild. This process has great significance for obtaining

tetraploid *D. floribunda* which may be expected to produce bigger tubers with higher diosgenin content. A competent embryogenic suspension culture of *D. opposita* has been obtained. Embryogenic callus was induced from stem segments cultured on an agar solidified M.S medium containing 2, 4 D for one month following placement as the embryogenic callus in a liquid medium containing 24 - D. The embryogenic tissue began to proliferate rapidly established suspension cultures consisted almost entirely of early stage proembryo with very little contamination from embryonic tissues. (Nagasawa et al 1989) The growth of *D. deltoidea* at high sugar concentrations was studied by Ball *at al* (1989). The ratio of fresh weight to dry weight of cells was observed to be dependent on the initial sugar concentration. However, it remained farely constant as long as the sugar was presant in the growth medium. Mitchell *et al* (1995) studied the effect of explant source, culture medium strength and growth regulators on the in vitro propagation of three Jamaican yams, i.e. *D. cayenensis,* D. trifida and D. rotundata. The results suggest that modal segments excised from young fast growing lines of these species are the best explant source for the purpose of commercial micropropagation.

Ravishankar and Grewal (1940) considered that AT Pase activity was an index of growth capability of cultured cells of *Dioscorea* and the character could be used as a marker for screening cell lines for improvement of growth in *Dioscorea* Browing of explants and callus tissue interrupts the growth of callus and its redifferentiation in tissue culture of chinese yam, *D. opposita.* Culture methods and conditions were examined by Kuginuki and Nishimura (1989) to prevent the browning of cultureed tissue of the plant. Browning of callus tissue was observed to be suppressed with vigorous growth in liquid medium in vertically rolated culture. The callus induced from axillary bud or young male influorescence showed little browing compared with that of others. When plant growth regulators was added to the M.S. medium callus growth was opitimum and the incidence of browing was the lowest at 2,4 -D and B.A. Addition of cytokinin in the medium enhanced the browning of the callus. Twyford et al (1990) worked out characterisation of the yam of *D. alata, D. cayenensis, D. rotundata, D. esculenta* and *D. bulbifera,* during tissue culture using isoelectric focusing of peroxidase and acid phasphatase isoenzymes.

While the diosgenin is synthesised particularly in the growing shoots of *Dioscora*, the tuber is only the storage organ for diosgenin and does not synthesize it (Bennelt *et al* 1963; Barker *et al,* 1966). However dispelling this belief, Chaturvedi, and Srivastava (1976) for the first time demonstrated with *D. deltoidea* that even tuber tissue could biosynthesize diosgenin. Initially, the percentage of diosgenin was 0.7, which was stepped up to more than two fold i.e. 1.6 by making certain changes in the composition of medium but with out adding any precursors.This is perhaps the highets percentage of diosgenin reported from *Dioscorea* callus cultures without feeding precursors. The two fold increase in fresh weight of callus in 2 months obtained in this study is also the highest record so far (Chaturvedi, 1970). It may be mentioned that earlier, diosgenin production from cultures, obtained by callusing the entire seedlings of several species of *Dioscorea* has been reported. (Kaul and Slaba, 1968; Mehta and Staba 1970). High content of diosgenin and fast rate of growth of callus as obtained by Chaturvedi (1979) hold promise for its large scale cultivations in fermentors and continous production of good amount of diosgenin. Besides the tuber callus, the callus tissue of leaf, stem and root of *D. deltoridea* were also grown satisfactorily. Vanek *et al.* (1999) studied biotrans formation of terpenoid. censtituent other than diosgenin in *D. deltoidea.* The leaf callus of *D. floribunda* showed so far the highest rate of proliferation ic 60 told increase, in fresh weight in 2 months, whereas the tuber callus of *D. prazeri* was grown to a limited extent Chaturvedi and Choudhuri (1980) marked that tuber callus of *D. deltoidea* showed prolific growth on a modified Schenk and Hild brandt's agar medium, a combination of 2.4. D and IAA when the callus synthenized diosgenin (1.6%) during 60 days of incubation. Suthar et al (1980) showed that 2, 4 D ; 2, 4,5 T in combination with GA3 and kinetin enhanced diosgenin contents in callus tissues of *D. assyptica* considerably. High concentration of growth hormones restricted callus growth. Ishida (1988) improved diosgenin production in *D. deltoidea* cell culture by immobilization in polyurethane foam cubes. Inmobilization increased the total diosgenin production by 40%. Increased efficiency in diosgenin production was greatest in 3% sucrose, high concentration inhibited diosgenin production. Lipsky *et al.* (1989) identifical diosgenin during in vitro tissue cultures of *D. caucasica.* Paukev. (1988) analysed diosgenin in cell culture

of *D. deltoridea*. Sengupta *et al* (1987) studued the behaviour of chromosomes and production of diosgenin and sterol in different hormonal regimes during different phases of callus growth of *D. floribunda* An increase in the amount of phytosterol was noted in culture during morphological differentiation. Sengupta (1989) also observed steroid formation during morphogenesis in callus cultures of *D. floribunda*. Roberts *et al.* (1989) studied diosgenin formation by freely suspended and entrapped plant cell cultures of *D. deltoidea*. A low rate of O_2 supply completely inhibited formation of diosgenin. A high O_2 supply rate led to the greatest formation of diosgenin in 30g/l sucrose. Entrapped plant cell culture with polyurethane foam led to delayed development of a suspension culture and to the formation of significantly higher concentration of diosgenin. *D. deltoide*a callus exposed to blue, red or white light was observed to be superior to dark incubated culture interms of growth of tissues and diosgenin and sterol production (Ravi Shankar. and Grewal, 1990). Renard *et al.*(1991) observed carbohydrate and principal ion consumption and production of steroidal sapogenins by *D. villosa* cell suspension. Development of media for growth of *D. deltoidea* cells and in vitro diosgenin production has been established by Ravi Shankar and Grewal (1991) According to them nutrient stress i.e. dipletion of nitrogen increased the production of diosgenin.

The *Dioscorea* is a slow propagating plant and cannot cope with the demand for its diosgenin value. Thus, tissue culture, specially the rapid propagation could be practiced with the aim to produce high yielding variety of *Dioscorea.*

There are 2 lines of approach for development of tissue culture method for rapid propagation of plants. i.e. (i) Regeneration of plants through organ culture without the intervening callus formation i.e. clonal propagation. (ii) their differentiation from callus culture. The latter affords faster rates, but rates are variable because callus culture is genetieally unstable (D'amata-1977). *Dioscorea* is reported to be affected by virus disease (Ruppel *et al.* 1966). It would, therefore, be worth while to find out cultural condition for growth of its shoot spices.

CHAPTER - II

ECOLOGICAL SURVEY IN DARJEELING AND SIKKIM HIMALAYAS WITH SPECIAL INTEREST ON DISTRIBUTION OF DIFFERENT SPECIES OF *Dioscorea* AVAILABLE IN THE REGION

Introduction

Diosgenin , a natural source material from *Dioscorea* yam is the basic chemical from which several steroid hormones including sexhormones, cortisone, other certicosteroids and the active ingredient in the oral contraceptive pill are produced. The total synthesis of diosgenin is not possible at the present state of knowledge . Although direct synthesis of a few finished steroids has been achieved,partial synthesis from naturally occurring compounds is still considered more economical in most cases. The consensus amongst chemist is that diosgenin will continue to be the favoured raw material at least for corticosteroids for many years to come (Bammi and Randhawa 1975).

Dioscorea is a genus of over 600 widely distributed species all over the world. In India,out of about 50species, *D.deltoidea* and *D. prazeri* have been identified to contain diosgenin in amount which are commercially feasible to extract. *D. deltoidea* contains higher quantities and has been the main species whose collection and processing has been undertaken by the various drug companies. However, the natural resources are limited and due to excessive collections the available supplies have been exhausted to a considerable extent. In many areas almost complete eradication has occurred. There have been practically no efforts to replant either by the drug companies or by the Forest Departments. The regereration time in nature of this species is fairly long. Starting from a small tuber piece of 50 gm or so it may take 7 -10 years before the new cycle of harvest could be initiated. The depletion of natural forests is

also reflected in the high price of the drug. Not only in India the picture of limited supply of diosgenin in has been reflected all over the worldal though according to Martin (1972), the recent fluctuations in supply have had social and political rather than agricultural causes. Very recently it has been reported *D. deltoidia* has become, threatened species in India (Mamgari *et. al.* 1998) and in Darjeeling district, West Bengal, (Samanta and Das, 2000).

As a result a search for alternative raw materials has been motivated by various reasons (Mann 1978). On account of the tremendous variability in climate and geographical features,the Darjeeling and Sikkim Himalayas is a veritable emporium of medicinal and other plants. Due to tremendous variation in altitude, differences in the aspects of climate within the hill areas vary greatly. Indeed these variations are very well marked in between hills and plains.

From time immemorial the local people in the region has been using different species of *Dioscorea* growing in their surrounding to alleviate physical illness in the from of extract ,decoction , powder of the under ground part of the species.

Besides the yam or underground part of some of the species are being utilised by the poor people as vegetable. As a result of collection and supplies of these materials from their natural habitats for the last several years have reduced their natural populations to a very scanty level.

Thus investigation has been carried out to survey the availability of different species of *Dioscorea* in various ecological condition of Darjeeling and Sikkim Himalayas with special interest on understanding the potentialities of people, forest and agriculture.

Materials and methods

Materials

Plants of *D. alata.* L. *D. kamoonensis* Kunth *D. arachidna* Prain and Burkill, D. sikimensis Prain and Burkill. *D. bulbifera* L., *D. esculenta, D. sativum* L. *D. prazeri* Prain and Burkill. soil smaples at diffrent places in the region.

Description of different Species of *Dioscorea*

1. *D.alata* L.

Plant quite glabrous,twining to the left yams, cylindrical, apical part much broader, gradually becomes narrow at the basal region, brown with rough surface, some vertical scars are present on the enter surface. Roots, present all over the yam with an average, length of 10.8 cm, Bulbils, round to cylindrical and lobed, brownish scars on the entire surface. Stem, acutely angled having 3 - 4 wings. Leaves, simple, opposite superposed, 7.5 cm – 18.0 cm x 3.8 cm - 12.5 cm, broadly ovate, cuspidately accuminate, 7-9 nerved along the base, petioles 5.0 cm to 12.5 cm long. Inflorescence, panicle, Capsules 2.5 cm X 3.8 cm, semicircular, retuse at the apex. Seeds with wing all round.

2. *D. kamoonensis* Kunth

Plants twining to the left, yams, round to oblong, dark brown to black, surface smooth to rough, covered thickly with long slender roots, 0.5 cm to 7.1 cm in length. Bulbils, round to oblong in shape,brown surface rough, single eye shaped.Scar on one side of each-bulbil. stem circular in out line with small prickles dorsiventrally flattened, compound, 3- foliate. Inflorescence, Panicle, Capsule, oblong, seeds, smooth.

3. *D. arachidna* Prain & Burkill

Plant twining to the left, yam multilobed, brown, surface smooth, yellow resins comes out from the wound, whole surface covered with long roots having average length of 6.1 cm. Bulbils rare Stem spinous. Leaves, trifoliate, alternate, petiole, dorsiventrally flaltened. Inflorescence, mixed panicle.

4. ***D. sikkimensis* Prain & Burkill**

Plants twining to the left. Yams, cylindrical, brownish black, surface nearly smooth, covered with some slender roots, having average length of 2.0 cm. Bulbil, round, bilobed, brownish black with rough surface having a few sears. Stem, glabrous, 6 - winged. Leaves, simple, opposite superposed, petiole dorsiventrally flatened. Inflorescence, panicle

5. ***D. bulbifera L***

Plant twining to the left, yams, variable in size and shape, mostly cylindrical, light brown, surface smooth, white inside, fine roots covering the surface, average length of root 1.7 cm. Bulbil, yellowish brown, surface rough, warted, variable in size, 3.5 cm to 9.0 cm across. Leaves alternate, about 12.0 cm -17.0 cm X 8.2 cm - 11.0 cm; acuminate Flowers, heterophyllous, male spike, 7.0 cm - 12.0 cm, long clustered in panicles, stamens, 6. Female, spike.7.0 cm - 23.0 cm long. Capsules 1.7 cm. to 2.3 cm long, oblong, Seeds, winged at the base.

6. ***D.esculenta* Prain & Burkill**

Plants twining to the left. Yams round to cylindrical, light brown, surface smooth, covered with many slender roots of average length 3.3 cm. Stem, pubes cent circular in out line, Leaves alternate, simple, petiole, dorsiventrally flaltended. Inflorescence, panicle.

7. ***D. sativum* L.**

Plants twining to the right, yam round brownish black covered with slender roots. surface nearely rough inner portion yellwish in colour, numerous long tubular roots covering the entire surface, the average length of root 7.5 cm. Bulbil, round, brown to black in colour; surface rough; many glandular outgrowths on the entire surface with 1-3 eye like structures. Stem, glabrous, some membrane like structures on the outer surface. Leaves alternate broadly ovate cordate petiole dorsiventrally flattened male spike 8-20cm. Capsule membranous. Seeds with a broad basal wing.

8. *D. prazeri* Prain & Burkill

Plant twining to the left, Yam short stout branched finger like 10 - 11.5 cm, long and 1.5-20 cm broad. Brownish-black with young surface, inner portion white or cream in colour, Stem glabrous sniooth, Bulbils not formed. Leaves alternate cordate or long cordate, gradually accuminate at the apex. The lobes at the base rounded, 15.5 cm long from the petiole to the apex of the leaf, 7 nerved, Petiole shorter than blade, Male flower sessile, spike like inflourescence 20.0 cm in length, slightly winged, Female flowers 18-20 in number, axis 20.0 cm in length, Seeds ovate oblong.

Methods

The work was carried out on the following lines of investigations.

Survey in connection with the accumalation of various information.

Information in connection with physiography, drainage, soil, climate human population and land use have been carried out after being contacted with the local people, office of grampanchayat, local police stations, government offices, specially the Directorate of Agriculture and Forests and local tea gardens.

Metoriological data was collected specially from the record of tea gardens and meteriological stations at Siliguri, Gangtok, and Calcutta (Alipur). Information collected after survey were compiled and worked in collaboration with the National Atlas and Thematic Mapping Organisation, Government of India.

Collection and identification of different plant materials

Collection of various plant materials was performed spicially during the study on vegetation. Proper identification of collected materials was done after comparing the prepared herbariums sheets with those available in various herbarium section of North Bengal University. Botanical survey of India, Gangtok, Sikkim, Loyed Botanic Gardens in Darjeeling, Central

NationalHerbarium (Botonical survey of India) Howrah) and Indian Museum Calcutta.

Collection soil of samples and their analysis

Soil samples were collected (0-20 cm) from different uncultivated land of different places along the altitidinal gradient. They were analysed in collaboration with Indo-British Fertilizer Education project (Hindusthan fertilizer Corporation Ltd), Soil Testing Laboratory, Siliguri following the methods described by Black (1965).

Results and Discussion

Darjeeling and Sikkim Himalayas lies in that part of Asia where the great areas, China , India, Tibet and Bhutan are very close together. (Fig.7,8) Though these areas have distinctive people , climates,plants and animals but types appear in common and tend to intermingle occassionally where the bounderies of different great areas merge together. Darjeeling and Sikkim Himalayas is roughly bounded by Nepal on its West flank, China - Tibet on the north and major part of the east. A part of east is bounded by Bhutan and West Bengal is on the South.

The region under study includes, the Sikkim the smallest hill state of the Indian Union, lying between 27^0 and 28^0 N latitude and 88^0 and 89^0 E longitude and Darjeeling district,the only hilly district of West Bengal ,lying between 26^0 32' and 27^0 13'N latitude and 87^0 59'and 88^0 53 ' E longitude. The mountaineous terrain of Sikkim consists of a tangled series of interlacing ridges rising ranges above range with the elevation varying from 300 m to 8400 m. The state is almost rectangular as compared to the Darjeeling district which is triangular in shape.

Inspite of the confused nature of the terrain pattern certain well marked features can be observed. In the north west of the district the lofty Singalila ridge which culminates ultimately in the lofty height of the Kanchanjungha (8,585 m) in Sikkim, enters the district forming the highest part of the district.

At Phalut in the district ,the ridge is nearly 3159m and it descends gradually to towards Sandakphu and further South at Maneybhanjan (1580m). The ridge continues South ward to the level of the plains near Mechi river. From Singallia the Senchal- Maldhiram spur just out in the eastern direction and moves in a N- NW to S -SE. alignment. -This spur is in the central part of the district and acts as a mountain knot from which as many as seven spurs diverge in different directions separating one valley from the others, the more prominent on the east side being the Takdah - Peshok ridge descending to the junction of the Rangit with the Tista and the Stiiong spur further South. Darjeeling town is on a spur running north from the Maneybhanjan , Senchal ridge which devides below the town into the Tukvar and the Lebong spurs before they descent to the Rangit river .(Fig.8).

The eastern ranges in the district are separated from theirWestern counter parts by the Tista gorge. East of the Tista , the highest ground is at the Rishila (2711m) the trijunction of Bhutan , Sikkim and India .From there one of the prominent ridges runs south -east and cuts off the Jaldhaka valley from the rest of the district. Another ridge descends lable just under1840m above the sea. From there an important spur leads south - west ward down to the plains and another north west of Rissium where it joins a ridge running north east to south west in pedong and the south western spur passes through Kalimpong and descends abruptly in the Tista Valley.

Thus the general physiography of the region to include slopping terrain of Sikkim (7, 096 sq.km) and Darjeeling (3,149sq.km) is the major feature over which most habitation , agricultural activity and forest cover may be found.Vertical range is from 100 m (foot hills) through 4000 m. (timberline)upto 8585 m (the Kanchanjunga peak). The total area of about 10,245 sq.km.is open to a greater extent at the southern part which constitute foot hills.

The most dominating river Tista rises in a glacier in North Sikkim 5526 m and drains the whole of Sikkim. It forms the boundary of Darjeeling district from the point where it is joined by the Rangpu down to its juction with the great Rongit flowing in from the West. It leaves the district at Sevok and

ultimately joins the mighty Bramhaputra river in Bangladesh. In Darjeeling district ,its principal tributaries are the Rangpu and the Great Rangit, the Riyang and the Sevok on the right bank. A part from Tista some of the major rivers which drain the hills are Balason, Rangbhang , Mahananda and Ranichhu. The Mahanda has its source near Mahabdhiram range to the east of Kurseong. The Balason rises near Lepcha Jagat on the Ghum - Simana range .On the extreme west is the Mechi river which becomes the part of the district boundary with Nepal. The Mahananda ,the Balason and the Mechi all flow into the Ganges. The rivers of the region are not navigable . They are mainly utilized for floating the long wood and generation of hydal power.

The geological formation of the region specially of the Darjeeling district consist of sedimentary rocks, confined to the hills on the South , and different grades of metamorphic rocks over the rest of the area. The great range was elevated during the Tertiary period on the side of an ancient sea the Tethys , that had accumulated sediments of different geological ages. The mountains are made of folded rocks and piled over another by a series of north South horizontal compression movement and thrusts. At many places the formation have been intruded by granites and the rocks of Peninsular India , which seem to have extended north wards as far as the Himalayan base. Frequently the strata within the range are inverted due to the over turning of the folds and their dislocation . Features of such inversion bringing the older beds above the younger one and characterise the whole length of the outer Himalaya. The Terai and the plains at the foot of the Himalaya were given their present form after the final upheaval of the range and consists of almost horizontal layers of unconsolidated sand silt, pebbles and gravel. The foot hills, north of the Terai,are made of similar but well cemented and more compact alluvial detritus . The rocks are of Tertiary age and have been included in the Naham stage of the Siwalik system of the outer Himalaya.

Resting over the Siwalik beds , is a group of still older rocks consisting of coarse , hard sandstone, sometimes solidified into quarzites of carbonaceous and splintery states of shales and of impersistent seams of powered coal . North of the Gandwana outcrops the hills are occupied by a group of low grade metamorphosed sediments represented by quartizites, slates, phyllites,

and foliated rocks composed of flaky minerals such as graphite etc. The Daling series rests under a variety of foliated and banded metamorphic rocks , partly sedimentary and partly igneceous origin. Those rocks are known generally as Darjeeling gneiss. On the journey between the plains and Darjeeling , the Tertiary beds crop out between Sukhna and Chunabati, the coal bearing Gandwanas below Tindharia, the Daling rocks between Tindharia and north of Gayabari and the Darjeeling gneiss over the rest of the distance.

Within its habitable portions, different social religions, linguistic and ethnic groups coexist practising different types of agriculture and pastoral activities. The population of Sikkim is 3, 16, 385 out of which 84% is rural and 16% is urban. The most significant feature with regard to settlement pattern is that the lower hills of the state are thickly populated with diverse economic activities while the high hills are inhabited by Lepchas in lower densities with self contained economics. This uneven distribution has resulted in more concentration of population in the east and south districts and lower concentration in the north district of the state. Lepchas are the aboriginal tribes of the state practising Buddhism and constituting 13% of the total population . Bhutias (14.1%) are distributed throughout the state and practise Buddhism. Bhutias settled in Lochen and Lachung of North Sikkim differ in many ways from Bhutias living in other parts of the state. They are called Lachenpa and Lachungpa Bhutias. The adaptation of these Bhutias to these high altitude region is linked to their different life style with regard to cultivation and animal husbandry. Presently 70% of the state population is constituted by Nepalese. They are industrious. Thrifty and excellent settlers. They rose to important positions in business and administration.With the exception of Sherpas and Tamangs, who are Buddhists, the Nepalese are Hindu by religion and have the usual division into castes. Brahamans rank highest in the caste and their main occupation is farming and government service. The Chhetris form a majority amongst the wealthy people of Sikkim. Tamangs are very skilled at a number of crafts while Pradhans are shopkeepers big business men and farmers. Sherpas are small land owners and also rear animals like sheep and mules. They are concentrated exclusively in few pockets of West Sikkim . The Gurung, Manager and Kirantis are essentially agriculturists

and live all parts of Sikkim. Five castes namely Kami , Damai, Lohar Sarki and Maji were classified as scheduled castes. The three main languages of the state are Nepali ,Bhutia and Lepcha, Hindi is understood by majority of the people.

The total population of Darjeeling district is about 10.24,369 . The district has an assemblage of various ethnic groups ,most of them being tribals. The great majority of the inhabitants in the hills are Nepalese, the rest is Chinese, Marwaris , Sherpas, Bhutias, Tibetans , and Lepchas in varying degrees. Each ethnic group has its own social and religious traditions so that there exists a great deal of cultural diversity in the district. Socioeconomically the district has rich natural resources. Good agroclimatic conditions of the areas have been utilised to develop only two industries like tea and medicinal plants including Cinchona.

The most important activity in the region is agriculture which occupies 81% of the working force. A sizable formation of the population are engaged for construction (3%)and services (6%).

Table 4. Meteriological data recorded in Darjeeling district, West Bengal, India

Month	Rainfall (mm)	Number of rainydays	Mean temperature (^{0}C)		Relative humidity	Sunshine hours perday
			Max.	Min		
January	35.4	9.1	15.8	1.3	89.8	4.1
February	40.1	13.2	16.2	2.5	90.1	4.0
March	51.2	12.3	19.7	6.2	89.1	3.9
April	204.1	16.1	22.1	8.1	88.2	4.2
May	250.2	26.2	22.8	11.5	92.2	2.8
June	701.4	29.1	22.5	13.8	95.2	1.4
July	835.5	30.0	22.1	14.2	95.7	1.0
August	680.3	29.0	22.7	14.5	95.5	1.4
September	560.1	26.8	22.8	12.8	93.6	1.8
October	85.4	16.1	23.8	9.5	90.7	4.5
November	9.5	4.5	22.1	6.5	87.5	5.8
December	7.2	3.8	18.7	3.2	89.0	5.2

Due to tremendous variation of altitude , differences in aspects ,the climate within the hill areas vary greatly. Indeed these variations are very well marked in between the hills and the plains . The three seasons identified for this region are summer (from April to June), the rains (July to October) and winter (from November till March) . The course of monsoonal winds strike the outer hills of singalila ridge and the high riding clouds enter the inner Sikkim hills reaching ultimately the Himalayan barrier in the north for the final flush. Rains and the high humidity which follow are the major climatic realms found in the region. (Table - 4, 5) A difference in the rainfall exists even in different areas of the same altitude depending on the nearby forest wealth and its composition. Near Gangtok, the annual rainfall is between 375 and 500 cm. While at Namchi located at the same altitude , the rainfall is about 200 cm. At higher elevation the climate is temperate and moist. Though the permanent snowline is above 5000 m. Often glaciers descend to 4000 m due to heavy snowfall effecting the over all climate of Sikkim by lowering down the temperature considerably (Venu et al,1990).

The soil of the region shows multiple varieties ranging from red - clay and sandy loam to grey - brown forestsoil. The forest soils of the hills are rich in organic matter being developed under forest cover act as cushions on rain water. The soil is eminently suitable for tea ,cardamon ,citrus fruits and few other crops. In the hills , the cultivators recognise only three kinds of soil: white , black , and red, of these the black soil is the richest , the white the poorest the red soil occupies an intermediate position, requiring heavy manuring to find as good as out turn as the black.

Table 5. Meteriological data recorded in Sikkim, India

Month	Rainfall (mm)	Number of rainydays	Mean temperature (°C)		Relative humidity	Sunshine hours perday
			Max.	Min		
January	40.1	11.5	10.1	5.0	87.8	2.01
February	80.5	14.8	12.5	6.1	87.0	1.8
March	110.8	17.1	17.1	9.8	83.8	2.1
April	223.5	18.5	20.9	12.5	75.8	2.7
May	615.5	28.6	21.3	14.6	89.0	2.4
June	532.8	28.4	21.7	17.4	94.6	1.3
July	658.5	30.1	21.7	17.3	96.2	0.9
August	625.6	30.1	21.8	17.2	95.8	0.8
September	395.5	27.5	21.3	16.4	95.0	1.5
October	216.5	17.2	19.2	13.3	89.0	2.1
November	54.5	8.2	16.5	9.9	82.4	2.9
December	13.9	5.0	13.2	6.7	82.8	1.7

The red earth and yellow loams of the foot hills are residual in character. The riverline alluvium developed at a distance is suitable for the production of rice, oilseeds,wheat, pulses and jute.Thus at lower and midhills rice wheat and millet are grown while at high hills maize ,barley and potato are the main orops. Among the horticulturalcrops guava, lime, lemon, ginger and oranges are restricted to lower and mid hills while high hills are the apple, plum peach, etc.

Table. 6 : Some characteristics soils at diferent altitudes of the Darjeeling and Sikkim Himalayas.

	Altitude (m)	pH	organic matter (%)	K ppm	P ppm	Fe ppm	Zn ppm	Mn ppm	Cu ppm
Rajaramo-hupur	Foothill	5.2	1.75	5.7	8.5	105.8	0.50	1.20	0.08
Sukhna	161	5.2	1.61	9.2	9.0	110.0	0.68	2.0	0.10
Rangpo	424	5.1	1.54	9.8	14.1	89.8	0.80	7.5	0.20
Kalimpong	1160	5.2	0.90	16.3	12.1	99.0	0.79	10.2	0.40
Turung	1300	5.8	0.86	13.8	27.5	90.0	1.9	5.8	0.51
Kurseong	1475	5.1	0.93	14.0	10.5	89.0	1.77	3.9	2.1
Namthang	1500	5.3	0.90	18.7	27.7	94.2	1.80	8.0	1.60
Nagi	1700	5.0	0.98	16.3	12.1	105.3	1.70	10.3	1.80
Gangtok	1800	5.0	0.90	18.2	13.1	72.5	1.85	3.7	0.85
Sonada	1971	5.0	0.97	17.5	20.0	84.6	1.98	4.5	1.50
Pernayangtse	2085	4.9	1.10	15.0	15.0	52.1	1.80	12.6	2.10
Darjeeling	2123	5.0	1.20	14.70	14.0	63.8	1.79	11.0	0.98

All the soils used for the study (Fig.9, Table 6) are acidic in nature (pH 4.9-5.8) In general the acidity of the soil tends to increase with altitude. This is not unexpected because of higher rate of leaching as a result of heavy rainfall at higher altitudes.

The percentage of organic matter increases with the increase is altitude of the locality for collection of soil samples. This is probably dueto the fact that the gradual change in the temperature and moisture favours the formation of humus like substances. However the presence of very high percentage of organic matter at foot hill soils as compared with the hill soils indicates that the former soils act as initial sink to the organic matters from the hills (Chakravorty and Chakravarti, 1980)

The available potassium and phosphorus content of all the soil is from low to medium (Table 6). The range of available concentrations of Fe, Zn Mn and Cu are 52.1— 110 ppm, 0.5-1.98 ppm, 1.20 — 12.6ppm and 0.08— 2.10ppm respectively. From the table 6 it appears that concentration of Fe increase in the soil having highest percentage of organic matter.

According to Sauchelli (1969), this organic matter forms soluble complex with iron and is easily available to the plants, the positive relationship between available iron and the organic matter points to increased availability of iron with increased organic matter. On the other hand the concentration of zinc is low in the soil of lower altitude having high percentage of organic matter.

According to lindsay (1972) organic matter can react with zinc in two important ways. Firstly, zinc from soluble mineral matter can be mineralised and made available. Secondaly zinc can be bound to organic matter constituents that are immobile in the soil and constitute a fixation machanism by which zinc is not released easily.

As regards Mn, the concentration of it is low at lower altitude where the soil has high percentage of organic matter (Table 6). It is known that organic matter forms insoluble complexes with manganese so when present in high amount it may have an adverse effect on manganese availability.

Fig. 10 shows distribution of forests, glaciers and cultivated lands as found in a region of Darjeeling and Sikkim Himalayas. It is perhaps in fitness of things that forest conservancy and scientific forest management was initiated in the District of Darjeeling first among all the districts of West Bengal ,more than hundred years ago . Besides , most of the areas in Sikkim state , excepting a few places , remained in natural condition for a long time.

The forest bearing different species of *Dioscorea* in the district of Darjeeling Sikkim State can be classified into two broad groups (1) plain forests and (2) Hill forests.

1. Plain Forests:

The soil near the river in the district of Darjeeling and Sikkim state is mainly sandy. It turns to deep loam as one proceeds to the interior . Depending on this various types of forests are noted. The riverain forests are found in sandy soils near river beds. Most important among this type are *Acacia catechu* and *Dalbergia sisoo* forests found along the beds of Tista, Sevoke, Mahananda, Rokti, Balason, Mechi, Rongdonding and other rivers.

Most important among the plain forests are the excellent sal , (*Shorea robusta*) forests of the foot hills. Sal is gregarious but often it is mixed with *Schima wallichi, Chukrasis tubularis, Largerstroemia parviflora Amoora volituka.*

Besides sal forests, dry mixed forest with occasional sal are also observed, the dominating species belong to *Terminalia, Gmelina, Sterculia, Tetrameles*, etc. There is another type of forest (Wet mixed) which contain evergreen species like *Artocarpus chaplasha, Machilus* sp., *Amoora wellichi, Michelia* sp. and *Eugenia.* sp.

2. Hill forests

The Hill forests of the District of Darjeeling and Sikkim state can be divided into three classes :-

1. Lower Hill Forests upto 3,000'altitude.
2. The Middle Hill Forests from 3,000' to 6,000' altitude.
3. The upper Hill Forests from 6,000' to 9,000' altitude.

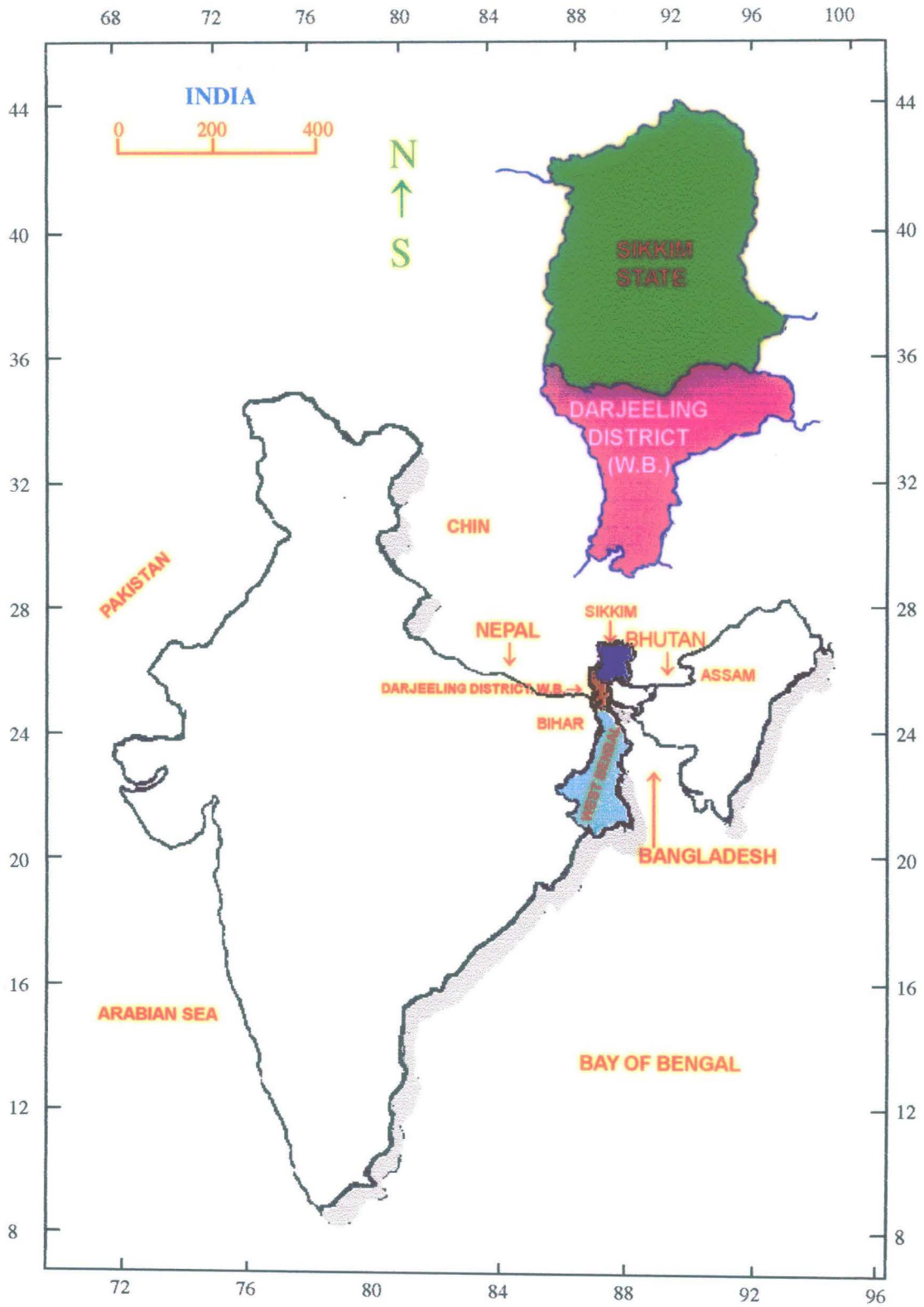

Fig.7 : Map of India showing Geographical position of Darjeeling and Sikkim Himalayas

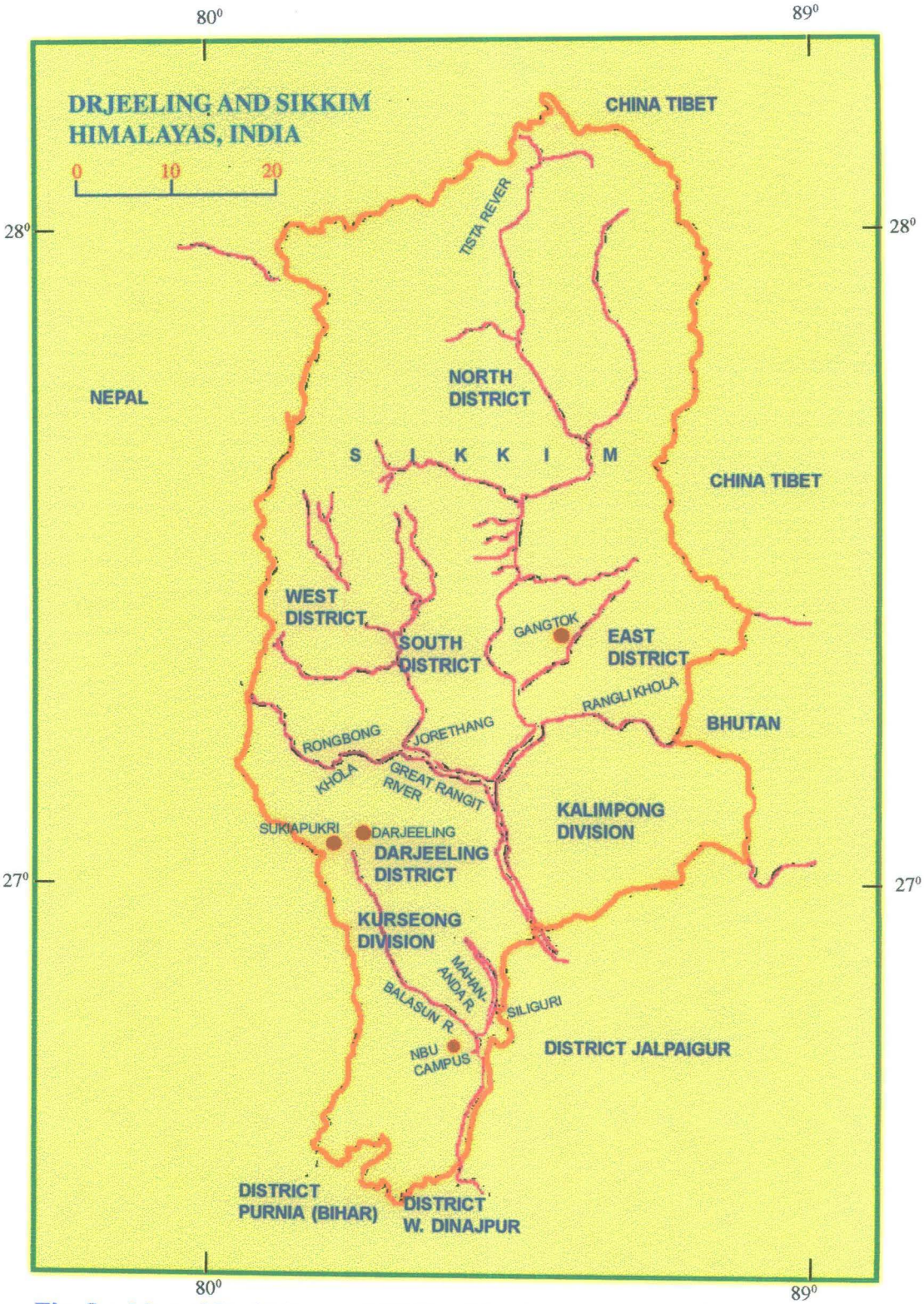

Fig. 8 : Map of Darjeeling and Sikkim Himalayas showing administrative divisions and distribution of rivers.

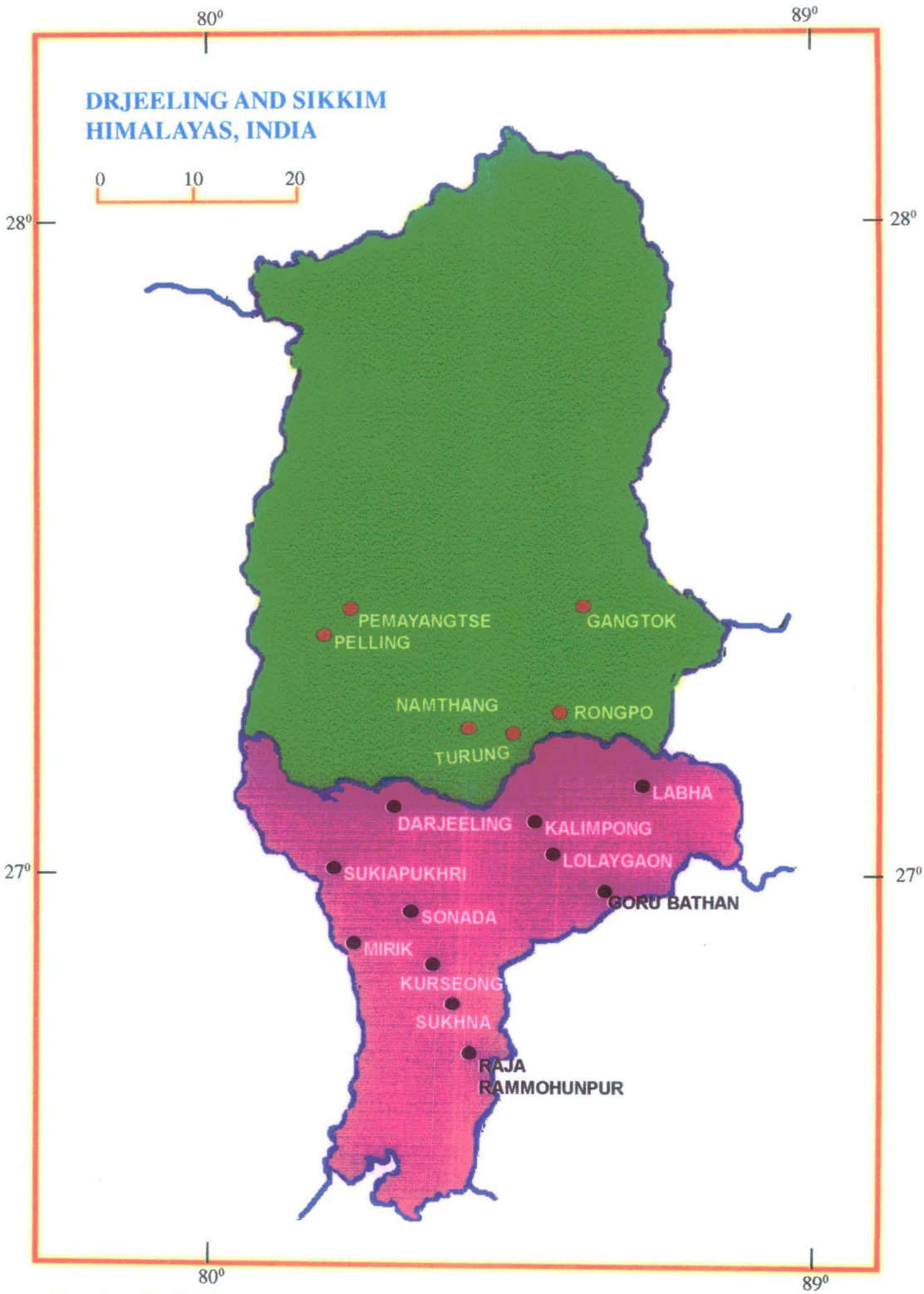

Fig. 9 : Darjeeling and Sikkim Himalays showing Geographical position of different locality for collection of *Dioscorea* **sp.** and soil samples.

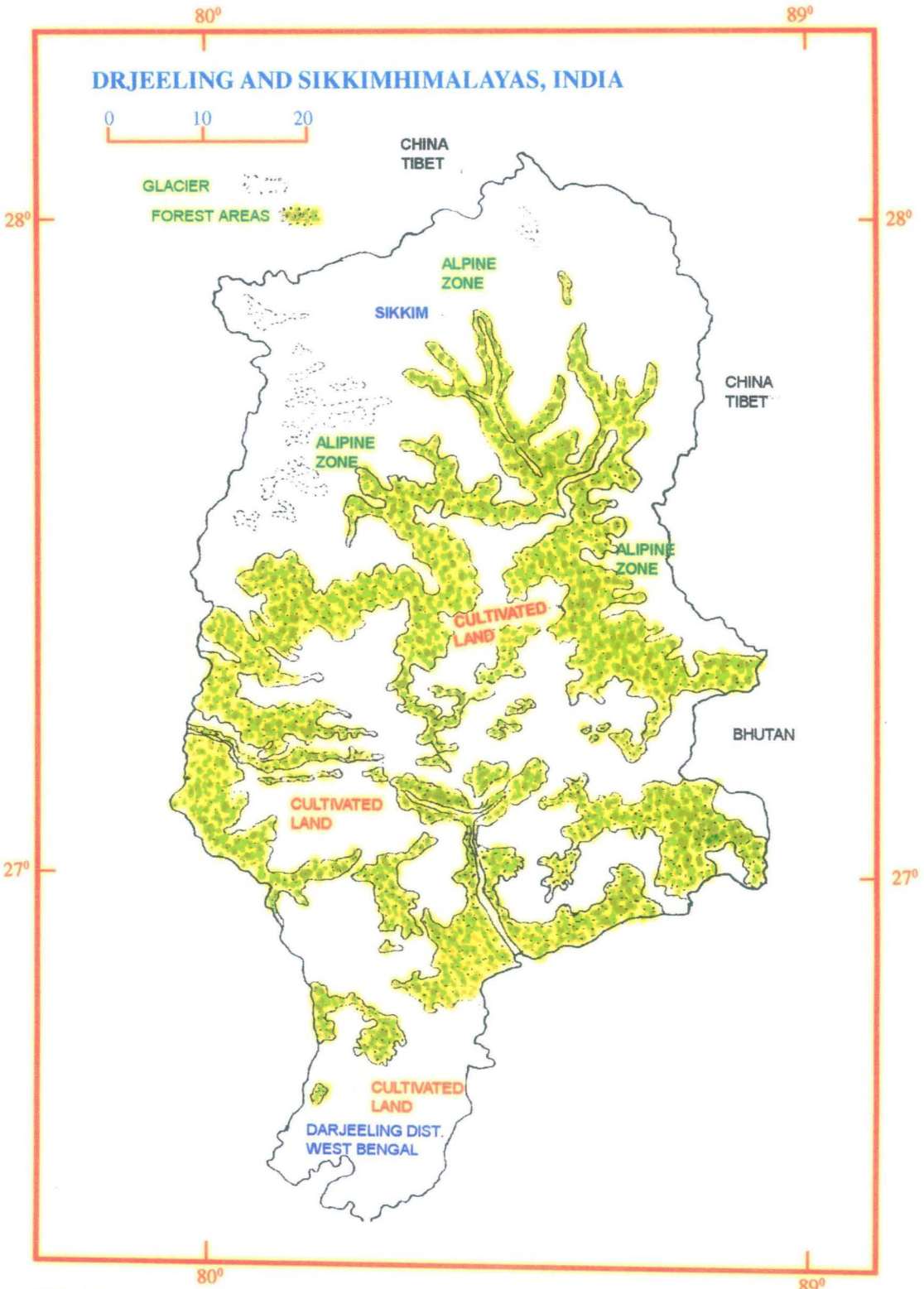

Fig.10 : Darjeeling and Sikkim Himalays showing lands distribution of forest and cultivated lands.

The Lower Hill Forests

In the Darjeeling Division, Sal occur on ridges and slope other than those with northerly aspects .In many parts it is gregarious but usually it is stunted and of poor growth with trees of all age classess present. Sal is found also in mixtures elsewhere with a large number of miscellaneous species of which paccasaj, (*Terminalia tomentosa*) Chilauni, (*Sachima Wallichii*) , Toon (*Cedrela toona*) and Chirrassi (*Chikrasia tabularis*) are the more valuable. In pockets or in northerly aspects the forests crop is varied in character, the chief species being champ (*Michelia* spp.) Panisaj (*Terminalia myriocarpa*), Gokul (*Alianthus grandis*), Simul (*Salmaliamal malbarica*), Lampati (*Duabanga sonneratioides*), Mainakath (*Terameles mudiflors*) Gamari (Gmelina aroborea), Mandani (*Acrocarpus fraxinifolius*) and Kadam (*Anthocephalus cadamba*) as well as the species mentioned above as in mixture with sal.

In Badamtam in the Rangit valley ,Pine (*Pinus longifoloa*) is found in fair quantity . This is the only locality in Bengal where pine occurs naturally.

The undergrowth consists of numerous herbaceous annuals and shrubs. Tuma bamboo (*Dendrocalamus hamiltoni*) grows extensively except on the driest slopes and forms almost pure bamboo forest in the moister areas. The "area abounds in climbers which do considerable damage to trees.The more common ones are Gurjo lahare (*Tinospora cordifolia*), Debre lahare (*Sparholous roxburghii*), Bhoria (*Bauhinia vahlii*) Kurkus (*Milletia pachyacarpa*) and Caechu (*Mucuna pruriens*), plantationsof Sal,Toon, Penisaj, Chikrassi, Lampati and recently Teak (Tectona grandis) have been formed in this zone.

In the Kurseong Division Sal grows pure or mixed with either deciduous species on ridges and on southern and eastern slopes of moderate gradient. Steep slopes are occupied by its deciduous associates, the chief of which are Paccasaj , Chilauni, Maina, Simul ,Karam (*Adina cordifolia*), Gamari, Harra (*Terminalia chebula*), Barrah (*Terminalia belerica*), Kimbu (*Morus laevigata* and Amla (*Phyllanthus emblica*), sal is rare over 2,500 feet altitude. Moist

land is occupied mainly by Lampati, Mandani, Toon (*Cedrela microcarpa*), Champ, panisaj, Malagiri (*Cinnamomum cecidodaphne*), Gokul, Katua, (*Castanopsis species*), Angare (*Phoebe hainesiana*) and Tejpat (*Cinnamomum tomala*).

In the Kalimpong Division sal is generally gregarious and occur on well drained slopes and ridges in the western and southern forests but owing to geological causes it does not occur east of the Chel river. Common and important species of this zone include Paccasaj (*Terminalia tomentosa*), Dabdabe (*Garuga pinnata*), Gamar ,Oodal (*Sterculia villosa*), *Chilauni,* Hatipaila (*Pterosperum acerifolium*), Tanji, (*Bauhinia purposea*), Lali (*Amoora wallichii*) and Ambake (*Eugenia kurzii*). Considerable areas are covered with the Tama bamboo. Common species in the undergrowth are *Phlogacanthus thrsiflorus, Doedalacanthus nervosus, Halmskioldia, sanguineanm, Tabernaemontana coronaria, Jasminum* sp. and various kinds of thorny climbers. The weeds *Eupatorium odoratum* and *Croton caudatus* - invade waste places and where there is a break in the canopy.

In the damper localities towards the eastern boundary of the district, Panisaj, Angare, Champ and Negeswar (Mesua ferrea) are common while in the rivedrin forests,i.e., in the sandy beds of the Lish, Chel and their tributaries ,Siris (*Albizzia drocera* and *odoratissima*), Khair (*Acacia cateche*) and Sisso (*Dalbergia Sissoo*) grow among the Kushila grass (*Saccharum imperatum*).

In Sikkim this zone of lower hill forest is characterised by heavy rainfall and the relative humidity is about 60% to 80% sal (*Shorea roleustr*) is gregarious on ridges and spus having well drained soils and is mainly confined to the southern and Eastern slopes of moderate gradient it grows in pure stands or mixed with such species as *Tectona grandis, Schima wallichii Mallotus philipineusis*. Among the shrubs spicies of *Clerodendron* are among lians, *Dioscorea, Simplax* are of common occurrence is the forest in Rangit vally Chirpine (*Pinus longifolia*) also occur along with *Shorea roleusta.* This zone is also characterised by the presence of wet mixed forest in the north and north eastern part of the state between Dickchu and Singlik and subsidiary

valleys of Talung, Rangrang and Dikchs. The common species in this zone is Jamun. (*Eugenia operculata*). *Dendrocalamus hamiltonii* is a common economic plant in the area.

The Middle Hill Forests

Forests of this zone are limited in extent in the Darjeeling Division. The chief species which occur here are the Alder or Uties (*Alnus nepalensis*), Walnut (*Juglans regia*), Birch or Saur (*Betula alonoides*), Pipli (Bucklandia populnea), Angare (*Phoebe* sp.) Mahwa (*Engelhardtia* sp.) Lekh toon (*Cedrela febrifuga*), species of Oaks (*Quercus*) the Spanish Chestnut (*Castanopsos*) and Chilauni (*Schima wallichii*) . Undergrowth is not heavy in this zone and consista of numerous herbs and shrubs . In certain areas the small pheling bamboo (*Pscudostachyum polymorphum*) is found. Plantations consists mainly of Panisaj, Toon, Pipli and Walnut.

In the Kurseong Division the main species are Lekh toon, Panisaj , Chilauni, Lampati ,Saur (Betula species), Kimbu, Angare (Phoebe attenuata), Sinkoli (*Cinnamomum* sp.) Malagiri, Mandani Siris, (*Albizzia* sp.), Mahwa (*Eangelhardita spicata*) and Champ (*Michelia* sp.) .

In the Kalimpong Division the upper limit of Sal is at 3, 000' in 3500' altitude. The number of species is fewer than at the lower altitudes , the principal being Chilauni , Katus (Castanops tribuloides and hystrix), Mahwa, Panisaj and often in gregarious Patches, Utis and Saur (Betula cylindrostachys) Large climbers festooning the forests occur naturally in this and zones below and above.

The zone of middle hill forests in the Sikkim state receives heavy rainfall and the relative humidity is above 75%. The winter is mild, followed by a comparatively dry seasons from February to April. The slopping area ensures quick drainage one striking feature of their forests in Namthang area is the extremely mixed dominance so that no single species cambs marked dominant. No tree species is to remain completely leafless for any period of the year. Thus tropical evergreen vegetation occurs in his particular zone. The important

species are *Castenopsis indica, C. trilenloides Ficus cunia, F. semicordata, Macaranga peltata, Symplocus paniculata Vitex negando Terminalia myrico earpa.* There is dense herbaceous ground layer consisting of *Hedyotis scandeus; Pilea innbrosa Leucas molisima.* etc. Among the climbers which are often gregarious in the forest and in fairly sunny forest opening, the outstanding spcies are *Thunbergia coccinea* T. lutea *Dioscorea alata. Mucuna macrocarpa* a large woody climber is also occasionally seen in this zone.

Upper Hill Forests

The greater part of the Darjeeling Division falls in this zone which is charactersed by a rather overmature stand of oaks, magnolias, and laurels. The species are numerous but the following occur in quantity : The Oaks, Buk (*Quercus lamellosa*) , Phalat (*Quercus lineata*) and Sungrekatus (*Quercus* pachyphulla), Katus (Castanopsis bystrix), Kawlas (Mechilus sp.), Champs (*Michelia* sp.),Ghoge Champ (*Magnolia campbellii*), Sinkoli (*Cinnaamomum* sp.) and Maple, Kapasi (Acer sp.), of the lesser species, Khankpa (*Evodia* sp.) Jhingani (*Eurva Japonica*) and Kharani (*Symplocos* sp.) are worth mention . The trees are stag- headed and covered with moss and lichens : the general appearance of the crop is poor, undergrowth is dense and contains many nettles, raspberries, ferns and bamboos, plantations in this zone are extensive and consist mainly of Dhupi (*Cryptomeria japonica*) introduced from Japan in the late 19th centurty, Utis, Pipli, Champ and the Oaks.

In the Kurseong Division, Saur (*Betula* sp.) is the only valuable indigenous species growing in the lower part of the zone.Toon (*Cedrela* sp.), Panisaj and Kimbu are found in small quantities. Certain parts of the zone suit Walunt and Pipli, . In the upper part, the principal valuable species are Buk ,Phalat , Champ (*Michelia excelsa*), Lalkawla (*Mechilus odoratiszsima*), Pipli and Lekh toon. Undergrowth is mainly Rubus, strobilanthes, bamboo and ferns , Lichens cover the boles and branches of trees.

In the Kalimpong Division Katus (*Castanopsis* sp.) becomes more common as one rises through the zones . Other species such as Tite Champ (*Michelia cathcartii*) . Malata (*Macaranga pustulata*), Tarsing (*Beilschmiedia*

sikhimensis) appear and gradually give place to Kawla (*Machilus gammieana*), Pahale (*Litsea elongata),* the Oaks (Quercus lamellosa), Champ (*Michelia excelsa*) Kapasi (*Accr campbellii*)and Kharani (*Symplocos the folia*). A few walnut trees are found. The commonest undergrowth are the bamboo (*Arundinaria racemosa*), wild raspberries and various species of *Strobilanthus.*

The zone of upper hill forest is characterised by heavy rainfall, moderately cold temperatures and high humidity spread over most of the year. Being cloudy for most of the time, incident sunlight is less. There is noticeable differences between summer and winter, ground forest being common in December and January. These wood lands of climatic climax-forest are seen scattered in deep valleys and slopes. The tress are generally of bushy appearance and shorter than those of tropical zone. The leaves are generally simple, smaller in size and often toothed, but at the same time firmer and leathery. The trees layers have less distinguishable strata. The reduction in tree strata coupled with a decrease in the height of surface canopy is associated with the increase in altitude. The shrubs and herbs are well marked but these moist loving forests are characterised by the rarity of lians and the predominance of epiphytes mosses and liver warts are in abundance the usual tree of upper storey are *Engelhardtia spicta, Lithocarpus elegans, Exbuklandia populnea, Alnus nepalensis Betula alnoides Castenopsis hystrix Ficus hookeriana, Schima wallichii, Oestodus paniculatus.* The second storey is mainly compared of *Vibernum erubescence, Quercus glauca* Styrex serrulatum. *Symplocus racemosa.* Tree ferns are very common in these regions.

The shrubby species which are found in abundance are. *Dichroa febrifuga, Melastoma malakath ricum. M. normale, Osbeckia stellata Oxyspora paniculata Eurya acuminata Clerodendron colebroo kianum.* The forest floor has a dense cover of, fungi mosses, ferns, Selaginella, Lycopodiumal other herbaccons as giasperms. *Myriactis nepaleusis, Senccio cappa S. Wallichii, Impetiens decipiens, I, arguta Didymocarpus albicalyx Pilea anisophylla, P. ternifolia, P. umbrosa* Numerous species of terrestrial orchids suchas *A nectochilus* sp. *Arachinis* sp. *Calanthe* sp. *Habenaria* sp. are common. The

altitudinal distribution of eight species of *Diascorea* available in Darjeeling and Sikkim Himalayas have been represented in Table 7.

Table 7 : Altitudinal distribution of different species of *Diescorea* availablein Darjeeling of Sikkim Himalaya.

Species	Altitudinal zones			
	0-3000ft	3000-6000ft	6000-9000ft	9000ft and above
D. anlata	—	—	—	—
D.kumaoneusis	—	—	—	—
D. Arachidna	—	—	—	—
D. Sikimensis	—	—	—	—
D. bulbifera	—	—	—	—
D. esculenta	—	—	—	—
D. sati uum	—	—	—	—
D. prazeri	—	—	—	—

Dioscorea is a genus of over 600 widely distritubed species. Some of the species like *D. alata* and *D. esculenta* have been cultivated for a long time for their edible tubers. There are about 15 species of this genus which are known to contain steroidal sapogenin. In India out of 50 species. *D. delteridea* and *D. prazeri* from North Western and North Eastern. Himalaya respectively have been identified to contain diosgenin in the amount which are commercially feasible to extract. The natural resources are limited and due to excessive collections the available supplies have been exhausted to a considerable exter. In many areas almost complete eradication of the species has occurred (Bammi and Randhawa, 1975).It has been reported earlier that *Dioscorea* species are distributed nearly through out India except in the dry north western regions. They require for their growth an annual rainfall of at least 30 inches. Their under ground parts can stand low temperature and they are found growing at elevations of 8000-15000 ft inthe Himalaya (Prain and Burkill 1936,1939).

Accroding to Hooker (1854) *D. alata* is a plant of tropical India. *D. alata* is cultivated practically in all the states in India. The recorded rainfall

in the regions where *D. alata* thrives is 60 inches per annum, distributed over the period of its vigorous growth. In areas of less rainfall it can be grown under irrigation. The methods of cultivation of *D. alata* vary in different areas. In parts of dry Deccan it is grown under irrigation in parts of Assam, Bengal, Behar and Malabar the growth is spontaneous and the crops need little attention (Wealth of India1952).

According to Hooker (1854) *D. kamoonensis* grows in temperate Himalaya at 4-6000 ft from Kashmir to Sikkim. The and Khasi hills at the altitude 5-6000ft. According to Prain and Burkill (1939) *D. kamoonensis* is distributed in N.W. Himalaya, Uttar Pradesh, Nepal Bhutan, Darjeeling, Sikkim, Assam Prain and Burhill (1939) pointed out the distribution of *D. arachidna* only in Assam (upto 4000ft). No report is available in connection with the distribution of *D. sikkimensis* in India.

According to Prain and Burkill (1939) *D. bulbifera* is distributed throughout India and specially in the Himalaya upto 6000ft. The species is a native of the tropies of the old world and occurs in rain forests extending from west coast of Africa to the furthest islands in the pacific (Welth of India, 1952). It is common throughout India ascending upto 6000ft in the Himalaya. It does not thrive in the drier parts of India. *D. esculata* is probally native is Siam and Indo China. It is cultivated in the moist tropical regions of Asia from Bombay coast in the West to the pacific islands in the east. In India, it is found in Malabar and Coromandal coast and in Deccan, Madhya Pradesh, Uttarpradesh, Biher, Orissa, West Bengal and Assam ascending upto 3000ft in eastern Himalayas. It occurs also in Khasi, Naga Garo hills and in Andaman (Wealth of India, 1952).

No report is available inconnection with the distribution of *D. sativum* in India.

D. prazeri occurs in the wetter parts of eastern Himalaya up to the altitude of 5000ft in North Bihar, North Bengal, Nepal, Sikkim, Bhutan and Abor hills and in Naga hills upto 5000ft. It prefers well drained soils particularly river banks (Prain and Burkill, 1936).

Summary

Investigation has been carried out to survey the availability of different species of *Dioscorea* in various ecological condition of Darjeeling and Sikkim Himalayas.

Much emphasis has been given on understanding the drainage pattern, physiography slopping, terrain and geological formation of the region.

Within its habitable portions, different social, religions, linguistic and ethnic groups, human population, the types of agriculture andpastoral activities have also been studied.

Meteriological data has been collected for understanding persent day climatic conditions in the region.

The characteristic features of soils of twelve different places at different altitudes in the region have been studied.

Altitudial variation of vegetation with special emphais on distribution of eight species of *Dioscorea* i.e. *D.alata, D. kamoonensis, D. arachidna, D. sikkimeesis, D. bulbifera, D. esculenta, D. sativum* and *D. prazeri* have been worked out.

CHAPTER - III

PHARMACOGNOSTIC STUDY OF DIFFERENT SPECIES OF *Dioscorea* AVAILABLE IN DARJEELING AND SIKKIM HIMALAYAS

Introduction

The genus *Dioscorea L.* (family Dioscoreaceae) comprising temperate as well as tropical forms is an important group of plants having economic importance. The tropical dioscoreas have of late attracted considerable attention because of their diosgenin content and growing demand for steroid sapogenins. Recently identification of the cyanoglucosides in economically important yam of different species of *Dioscorea* has been worked out (Mullinge et al. 1999).

All together eight different species of *Dioscorea* have been collected from different localities in the region of Darjeeling and Sikkim Himalayas to work out whether they could be utilised as alternative source of diosgenin. It is very interesting to note that out of all these species some are edible and others are poisonous. Yet a few of them have been deserved to be very much effective against various diseases of domestic animals. Plant parts of these dioscoreas resemble so much in their external appearance that it is very difficult to identify them properly. Thus it is necessary to investigate the species from pharmacognosy point of view to work out certain characters for their identification.

It was not until 1815 that the term "Pharmacognosy" was introduced by C.A.Scydler, a medical student in Halle/Saale, Germany. It is derived from two Greek words "Pharmakon", drug and "gnosis", knowledge. For a long time pharmacognosy is being applied for botanical and chemical identification of drug. In a broad sense, pharmacognosy embraces a knowledge of the history, distribution, cultivation, identification, evaluation and use of drugs and economic substances affecting the health of man and animals.

Besides these, organoleptic study of the plant is a part of the programme of pharmacognosy. This particular study refers to evaluation of drug by means of various sense organs of human being for realisation of taste, odour, colour, shape and also for understanding the feeling by touch. Pollen morphology of *D. prazeri* and *D. bulbifera* has been worked out by Samanta and Das (2000).

The present study is accordingly intended to highlight, morphological, anatomical, organoleptic and chemical characters of different plant parts of dioscoreas so that all these characters may be utilised for their rapid identification.

The observations on this part of work has been, represented in two sections.

Section (A) :

Organoleptic, macroscopic and microscopic study on different plant parts of *Dioscorea sp.*

Section (B) :

Phytochemical investigation on different *Dioscorea species* with special emphasis on steroid constituents.

Section (A) Organileptic, Macroscopic and Microscopic Study on Different Plant Parts of *Dioscorea* sp.

Materials and Methods

Materials : Stem, root, yam and leaf of eight species of *Dioscorea* i.e. *D. alata, D. kamoonensis, D. arachidna, D. sikkimensis, D. bulbifera, D. esculenta, D. sativum* and *D. prazeri.*

Methods

Organoleptic evaluation

Organoleptic study including macroscopic appearance of different species of *Dioscorea,* their shape, colour, feeling by touch, taste, marking and odour has been carried out following. Trease and Evans (1997).

Macroscopic evaluation

Anatomical preparations have been made and studied following Metcalfe and Chalk (1950) and Esau (1953). Plant parts were sliced, dehydrated and blocks were prepared in paraffin wax for microtomy following the schedule of Johansen (1940). Cameralucida drawings were prepared to highlight the diagnostic characters for quantitative microscopy. Leaves were cut into pieces, boiled with absolute alcohol to eliminate chlorophyll. Green alcoholic solution was decanted off and the opaque white pieces of leaf were heated in presence of lactic acid for 3-4 minutes and kept in hot condition for 10 min. The transparent piece of leaf was mounted in 50% glycerine and microscopical determinations were made following Trease and Evans (1997).

Result

Organoleptic and macroscopic observations

D. alata L :Stem, reddish green in colour, glabrous acutely angled. Root, distributed all over the surface of the yam with the length 20-34 cm. Yam, cylindrical, much broad at the proximal end 7-9 cm, average length 42 cm, but gradually becomes narrow (2.5-3 cm) at the basal region, brown with rough surface, hairs present, some vertical scars present on the outer surface. Leaf, simple, green dorsiventrally flattened, opposite superposed, 7.5-18.0 cm x 3.8-12.5 cm, broadly ovate cuspidately acuminate or subhastately cordate, 7-9 nerves, margin entire, slightly notched at the base with rounded lobes directed outwards.

D. kamoonensis Kunth :Stem, green, cylindrical with small prickles, root present thickly all over the surface of yam, long slender, 0.5-7.1 cm in length. Yam, oblong, length 10-26 cm, and diameter 2-4 cm and 6-14 cm the basal and apical part respectively, dark brown to black, surface smooth to rough. Leaf, green alternate, petiolate, dorsiventrally flattened, compound, 3-foliate, leaflet, ovate, apex acuminate, margin entire.

D. arachidna Prain & Burkill : Stem, light green, cylindrical, spinous. Root, distributed all over the surface of yam with an average length of 6.1 cm. Yam, round, diameter 15-18 cm, brown in colour, surface smooth, yellow resin

present. Leaf, light green, compound, trifoliate, alternate, dorsiventrally flattened, petiolate, 3.5-4.5 cm, long, leaflet, ovate, acuminate, margin entire.

D. sikkimensis Prain & Burkill : Stem, greenish red, glabrous, 6-winged, root, slender having average length of 2.0 cm covering the surface of yam. Yam, oblong much widen in the middle (diameter, 6.5cm), average length 10.5 cm. brownish black in colour, surface nearly smooth, leaf subhastately or deeply cordate, base with rounded lobes on both the sides of wide notch, green, simple, opposite, superposed, dorsiventrally flattened, petiolate, 3-4.5 cm. long.

D. bulbifera L : Stem light green, glabrous, cylindrical, distinctly ridged but not winged. Root, plenty, thin, average length of 1.7 cm. covering the surface of the yam. Yam, variable in size and shape, unbranched, mostly cylindrical, middle portion much broader than the remaining part, light brown in colour, surface smooth, leaf, opposite superposed, green, ovately acuminate, deeply cordate base with rounded lobes, directed outwards, dorsiventrally flattened, 12.0-17.0 cm x 8.2-11.0 cm, petiole, 4.2-7.1 cm.

D. esculenta Burkill : Stem, green covered wtih prickles, cylindrical, root, slender, average length of 3.3 cm, covering the outer surface of the yam. Yam, round to cylindrical, light brown, surface smooth . Leaf, green, alternate, simple dorsiventrally flattened, cordate, acuminate, margin entire highly notched at the base, petiolate 3-4 cm long.

D. sativum L :Stem, light green to brownish, nearly cylindrical, glabrous with some membrane like structures on the entire surface. Roots, numerous long, tubuler, average length 7.5 cm covering the outer surface of the yam. Yam round having an average diameter of 9.4 cm. brownish black with nearly rough surface. Leaf green, simple alternate broadly ovate or cordate, dorsiventrally flattened, petiolate, 1.5-2.5 cm. long, apex acuminate, margin entire slightly notched at the base round basal, lobes slightly divergent.

D. prazeri : Prain & Burkil :Stem, green, glabrous cylindrical with smooth surface, Root, slender covering the surface of yam. Yam cylindrical, branched, branches finger like tubular structure, 10-13 cm long average diameter 4.1 cm. grey brown or nearly black with rough surface. Leaf deep green alternate cordate

or long cordate, gradually accuminate at the apex, the lobes at the base round with wide sinus. More or less seven nerved, the first pair of nerves enclosing an elliptic ovate and the second pair embraces an ovate area, upper surface shining and smooth, lower surface glabrous with prominent nerves, margin entire, petiole shorter than blades, glabrous 4.8 cm. long.

Microscopic Observations

D. alata : T.S. of stem is elliptic in out line surrounded by highly cuticularised epidermal cell, compactly arranged, nearly rectangular having average length 20-25μ and breadth 15-17.5 μ. Epidermal hairs are absent. Cortex is made up of 4 to 5 layers of parenchymatous cell containing chloroplasts having diameter 25μ to 30μ. Solid pith is made up of parenchymatous cells, diameter 30μ to 40μ. Vascular bundles are arranged in one ring embeded in the sub cortical slerenchymatous band of pericycle, thickness 100 - 130μ forming a single hollow cylinder. Vascular bundles are oval, closed and collateral. Metaxylem shows diameter 50 to 80μ.

T.S. of root is circular in outline, surrounded by epidermal cells, length 50μ to 55μ, breadth 25μ to 30μ, broad cortex, 325μ in thickness is made up of parenchymatous cells. Endodermis is present. There is a very wide band (90 - 100μ) of pericyclic seterenchyma. Vascular bundles are arranged in two circular rings. A ring of xylem and phloem is embeded in the seterenchyma and the other is much away from the sclerenchymatous zone. The average diameter of metaxylem is 135μ. Idioblasts containing as ascicular crystals, length 50μ are distributed in the cortex and pith.

T.S. through the central region of yam shows that tissue is differentiated into epidermis, cork layers and ground tissue. The epidermal cell, length 25 to 27μ breadth 15.0 - 17.5μ are compactly arranged. The cork consists of numerous layers of thin walled, flattered cells, length 45μ to 70μ, breadth 28μ to 30μ. The length of ascicular crystals in some idioblasts, distributed in ground tissue is 70 to 75μ.

The upper epidermis of leaf in T.S. consists of polygonal, cells length 35 to 45μ breadth 25 to 35μ. Stomata are very rare on the upper surface of leaf

though these are present on the lower surface. In T.S. the tissue of mid rib projects slightly on the underside with an entire semi-circular outline. The vascular bundle is surrounded by a zone of sclerenchymatous tissue. Beneath the sclerenchyma zone there is an arc of parenchyma. All these cells excepting a circular patch of parenchymatous tissue contain chlotoplasts. The dorsiventral lamina is about 250μ thick with a single layer of pallsade cells, length 60 to 75μ, breadth 25 to 30μ and a spongy tissue of 3 to 4 layers of cells having diameter 25 to 35μ. Lower epidermis consists of cells length 13 to 15μ, breadth 10 to 12μ with a large number of stomata, lengths 48 to 50μ breadth 30 to 35μ. Stomata are generally of a monocytic type. Idioblasts containing ascircular crystals, length 50μ, are present alongwith the spongy cells.

In T.S. the outline of the basal region of the petiole is dome shaped in structure. An average of seven vascular bundles more or less equal in diameter are arranged in a circle. Hairs are present.

D. kamoonensis : T.S. of stem is circular in outline, surrounded by cuticularised epidermal cell, compactly arranged rectangular having average length 40 to 45μ and breadth 30 to35μ. Epidermal hair 115μ is present. Cortex is made up of 6 to 8 layers of parenchymatous cell containing chloroplasts having diameter 25 to 35μ. Solid pith is made up of parenchymatous cells, diameter 40 to 55μ. vascular bundles are arranged in two rings. A ring of vascular bundles are embeded in a hollow cylinder of sub-cortical sclerenchymatous band. of pericycle having thickness 60 to 75μ. The other ring of vascular bundles are much away from the sclerenchymatous pericycle but more towards the pith. Vascular bundles are oval, closed and collateral. The central vascular bundles are larger than the peripheral bundles. Each vascular bundle is surrounded by a few layers of sclenrenchymatous cells. Metaxylem shows diameter 100 to 110μ.

T.S. of root is circular is outline surrounded by epidermal cells, length 20 to35μ, breadth 12 to15μ. Cortex, 240μ in thickness and is made up of parenchymatous cells. Endodermis present. There is a thin layer (65 to 70μ) of pericyclic sclerenchyma. Vascular bundles are apposed to the pericyclic layer. The average diameter of metaxylem is 130μ. Idioblasts containing ascicular crystals, length 50μ are distributed in the cortex and pith.

T.S. through the central region of yam shows that tissues is differentiated into epidermis, cork layer (200μ) and ground tissue. The epidermal cells length 35 to 40μ , breadth 20 to 25μ, are arranged in a row. The length of ascicular crystals in some idioblasts, distributed in ground tissue is 50 to 55μ.

The upper epidermis of leaf in T.S. consists of more or less rectangular cells length 45-75μ, breadth 40-45μ. Stomata are very rare on the upper surface of leaf though they are very common on the lower surface. The vascular bundle is surrounded by a zone of sclerenchymatous tissue (35μ). The midrib projects much from the surface of lamina on the under side with an entire circular outline. The dorsiventral lamina is about 185μ thick with a single layer of pallisade cells, length 55μ to 70μ, breadth 20-30μ and a spongy tissue of 2 to 3 layers of cells having diameter 25 to 28μ. The lower epidermis consists of cells, length 23μ to 25μ, breadth 22μ to 24μ with a large number of stomata which is paracytic type. Idioblasts containing ascicular crystals are rare in orruraence.

In T.S. the outline of the the basal region of the petiole is semicircular in nature average of five vascular bundles of equal size in diameter are distributed along the periphery of ground tissue.

D. arachinda :T.S. of stem is circular in outline. It is surrounded by highly cuticularised epidermal cell, compactly arranged rectangular shape, length (30μ to 35μ, breadth 20μ to 22μ. Epidermal hairs (150μ) are present. Cortex is made up of 5 to 6 layers of parenchymatous cell containing chloroplasts having diameter 25 to 30μ. Solid pith is made up of parenchymatous cells, diameter 40μ to 45μ. Vascular bundles are arranged in two rings. One is embeded in the cortical sclerenchymatous band of pericycle, thickness 14μ to 15μ to form a simple hollow cylinder. The other ring of vascular bundle is distributed towards the pith. Vascular bundles are oval, closed and collateral. Metaxylem shows diameter 60μ to 100μ. Each vascular bundle is surrounded by one or two layers of sclerenchymatous tissue, 15μ to 18μ in thickness.

T.S. of root is circular is outline surrounded by epidermal cells, length 15μ to 20μ, breadth 14μ to 18μ cortex is broad, 105μ in thickness and is made up of parenchymatous cells. Endodermis is present. There is a wide band (180μ

to 225μ) of pericyclic sclerenchyma. Vascular bundles are embeded in the sclerenchyma zone. The average diameter of metaxylem crystals, length 35μ are distributed in the pith.

T. S. through cortical region of yam shows that the tissue is differentiated into epidermis, cork layer and ground tissue. The epidermal cell, length 15μ to 18μ, breadth 10μ to 12μ are compactly arranged. The cork (15μ) consists of numerous layers of thin flattened cells length 30 to 45μ, breadth 15μ to 20μ. The length of ascircular crystals in some idioblasts distributed in ground tissue is 40 to 55μ.

The upper epidermis of leaf in T.S. consists of polygonal cells about 45μ to 50μ by 30μ to 35μ. Stomata are very rare on the upper surface of leaf though they are present on the lower surface. The midrib is not projected from the surface of lamina on the under side. The vascular bundle is surrounded by a layer of bundle sheath. There are two patches of sclerenchyma on the upper and lower part of the vascular bundle i.e. sclerenchyma zone is not continuous surrounding the vascular bundle. The dirsoventral lamina is about 250μ in thickness with a single layer of pallisade cells, length 55μ to 75μ, breadth 45μ to 50μ. There are 3 to 4 layers of spongy tissue, the diameter of each cells is 35μ to 40μ, lower epidermis consists of cell having length 30μ to 35μ and breadth 25μ to 30μ with a large number anomocytic stomata (30μ to 35μ by 15μ to 18μ). In T.S. outline of the basal region of the petiole is more or less circular with a portion of flat arc. An average four large and four small vascular bundles are distributed along the periphery of the ground tissue. Hairs are present.

D. sikkimensis : T.S. of stem is more or less triangular in outline with a curved base with projected angular processes. It is surrounded by highly cuticularised epidermal cell, compactly arranged, nearly rectangular having average length 25μ to 28μ and breadth 13μ to 15μ. Epidermal hairs are absent. In general contex is made up of 3 to 4 layers of cells containing chloroplasts. The average diameter of each cell is 30μ. Solid pith is made up of parenchumatous cells having diameter 40μ to 50μ. Vascular bundles are arranged in two rings, one apposed to the subcortical sclerenchymatous band of pericycle (120μ to 140μ) to form a hollow cylinder. The other ring of vascular bundles are distributed

more towards the pith. Vascular bundles are oval, closed and collateral. Metaxylem shows diameter of 55μ to 110μ. Each vascular bundle is surrounded by a layer of sclerenchymatous cells.

T.S. of root is circular in outline, and is surrounded by epidermal cells, length 20μ to 30μ breadth 12μ to 15μ. Broad cortex 230μ in thickness is made up of parenchymatous cells. Endodermis is present. There is a very wide band (400μ to 425μ) of sclerenchymatous pericycle. Vascular bundles are arranged in one circular ring totally embedded in the sclerenchyma. The average diameter of metaxylem is 125μ. Idioblasts containing crystals, length 55μ are distributed in the cortex and pith.

T.S. through the cortical region of the yam shows that tissue is differentiated into epidermis, cork layer and ground tissue. The epidermal cells, length 12μ to 15μ, breadth 8μ to 10μ are compactly arranged. The cork (155μ) consists of numerous layers of thin walled flattened cells, length 30μ t0 40μ, breadth 12μ to 20μ. The length of the ascicular crystals in idioblasts distributed in ground tissue is 35μ t0 40μ.

The upper epidermis of leaf in T.S. consists of polygonal cells length 40 to 75μ and breadth 20 to 50μ. Stomata are absent on the upper surface of leaf though they are present on the lower surface. The mid rib projects very much from the surface level of lamina on the under side. The vascular bundle is surrounded by a layer of sclerenchymatous cells. The lamina is about 250μ in thickness with a single layer of pallisade cell, length 55 to 70μ and breadth 20μ to 50μ and 4 to 5 layers of spongy cells having diameter from 25μ to 35μ. Lower epidermis consists of cells with large number of stomata length 50 to 55μ, breadth 20μ to 30μ. Stomata are generally paracytic.

In T.S. the outline of the basal region of the periole is dome shaped with an average of five vascular bundles having equal diameter, are distributed in a circle in the ground tissue. Hairs are absent.

D. bulbifera : In T.S. the outline of the stem shows pentagonal characteristic with somewhat curved arms. It is surrounded by cuticularised epidermal cell compactly.arranged rectangular and having the average length 12 to 15μ and

breadth 6 to 10μ. Epidermal hairs having average length of 50μ are present. Cortex is made up of 3 to 5 layers of parenchumatous cell containing chloroplasts having diameter 20μ to 30μ. Solid pith is made up of parenchymatous cells having diameter 50μ to 55μ. Vascular bundles are arranged in two rings. the outer ring of vascular bundles are apposed to a sclerenchymatous band (30μ to 45μ) and which is pentagonal in gshape to form a hollow cylinder of pericycle. The outer ring of vascular bundles are more towards the pith. Vascular bundles are oval, closed and collateral and each is surrounded by single layer of sclerenchyma having thickness of 20μ. Metaxylem shows diameter 50μ to 90μ. Idioblast cell containing ascicular crystals length 18μ to 30μ are distributed in the cortex and ground tissue.

T.S. of root is circular in outline and is surrounded by epidermal cell, length 15μ to 35μ, breadth 12μ to 15μ. Broad cortex, 260μ in thickness is made up of parenchymatous cells. There is a narrow band (40 to 45μ) of pericyclic sclerenchyma. Vascular bundles are arranged in a ring and each vascular bundle, touches the sclerenchyma band only with the smallest part of protoxylem. In some of the vascular bundle there is a gap of parenchyma attached to protoxylem. Vascular bundle is surrounded by a thin layer of sclerenchyma. The average diameter of metaxylem is 215μ. Idioblast containing ascicular crystals, length 45μ to 55μ are distributed in the cortex and pith.

T.S. through the yam shows that tissue is differentiated into epidermis, cork layer, endodermis and ground tissue. The epidermal cell, length 20μ to 25μ, breadth 8μ to 10μ are compactly arranged. The cork (125μ) consists of numerous layers of thin walled flattened cells length 20μ t0 60μ, breadth 15μ to 25μ. The length of ascicular crystals in idioblasts distributed in ground tissue is 45μ to 50μ.

The upper epidermis of leaf in T.S. consists of polygonal cell length 30μ to 50μ, breadth 20μ to 35μ. Stomata are rare on the upper surface of leaf though these are present on the lower surface. in T.S. midrib portion, projects on the underside with deeply knotched outer surface. The vascular bndle is surrounded by a zone of sclerenchyma. The dorsiventral lamina is about 255μ in thickness with a single layer of pallisade cell having length 40μ to 60μ and breadth 25μ to

35μ and spongy tissue of 4 to 5 layers of cells having diameter of 25μ to 45μ. Lower epidermis consists of cell length, 15μ to 30μ breadth 12μ to 20μ. Stomata are generally paracytic type and having with length 45μ to 55μ and breadth 20μ to 30μ. Idioblasts containing ascicular crystals having length 20μ are present in the region of midrib and spongy tissue.

In T.S. the outline of the basal region of the petiole is pentagonal in shape. An average of ten to eleven vascular bundles are arranged in the circle, out of which big and small vascular bundle alternates with one another. Very small hairs are present.

D. esculenta : T.S. of stem is circular in outline surrounded by cuticularised epidermal cell compactly arranged, nearly rectangular and having average length 20μ tp 25μ and breadth 18μ to 20μ. The average length of epidermal hair is 10μ. Cortex is made up of 6 to 9 layers of parenchymatous cell containing chloroplasts having diameter 20μ to 35μ. Vascular bundles are arranged in two rings. A number of small vascular bundles are embeded in the sub cortical sclerenchymatous band (25μ to 30μ) of pericycle. An number of large vascular bundles are arranged in a ring quite away from the pericycle layer. Vascular bundles are oval, closed and collateral. Metaxylem shows diameter of 100μ to 170μ.

T.S. of root is also circular in outline surrounded by epidermal cell, length 30μ to 35μ, breadth 15μ to 20μ. Cortex of 145μ is made up of parenchmatous cells. Endodermis is present. There is a very wide band (300μ to 310μ) of pericyclic sclerenchyma. All vascular bundles are embeded in the sclerenchymatous zone. The average diameeter of metaxylem and protoxylem are 75μ and 40μ respectively. Idioblasts containing ascicular crystals, length 55μ are distributed mainly in the pith.

T.S. through the yam shows that tissue is differentiated into epidermis, cork layer and ground tissue. The epidermal cell, length 20μ to 30μ, breadth 15μ to 18μ are compactly arranged. The cork (225μ) consists of large number of layers of thin walled flattened cells length 25μ to 50μ breadth 20μ to 25μ. The length of ascicular crystals in some idioblasts distributed in ground tissue

is 45μ. The upper epidermis of leaf in T.S. consists of polygonal cells having length 35μ to 70μ and breadth 25μ to 45μ. Stomata are very rare on the upper surface of leaf though these are available on the lower surface. Stomata, are having with the length 25μ to 50μ and breadth 15μ to 20μ. Stomata are generally a mixture of paracytic and anomocytic type. The margin of subsidiary and other epidermal cell on the lower surface is sinuate. on the other hand the margin of the epidermal cell on the upper surface is entire. In T.S. midrib portion projects on the underside to form an entire oblong outline. The vascular bundle is surrounded by a sclerenchymatous cells contain chloroplasts. The dorsivntral lamina is 225μ in thickness with a single layer of palisade cell having length 100μ to 110μ and breadth 35μ to 125μ. Spongy tissue is made up of 3 to 4 layers of cell having diameter 25μ to 40μ. The length of lower epidermal cell has the range of 55μ to 90μ and that of breadth is 35μ to 55μ.

In T.S. the outline of the basal region of the petiole is circular in nature with an average of eight vascular bundles are arranged in a circle, out of which four are five. Multicellular hairs are present.

D. sativum : T.S. of stem is polygonal in outline with curved sides. It is surrounded by cuticularied epidermal cell compactly arranged, nearly rectangular having average length 15μ to 20μ , breadth 10μ to 18μ . Epidermal haris are present. Cortex is made up of 5 to 6 layers of parenchymatous cells containing chlotoplasts. Cortex is much broad to form a pyramidal structure at each angle of the polygonal outline. The diameter of cortical cell is 20 to 25μ. Vascular bundles are arranged in two rings. A ring of vascularbundles is apposed to a hollow cylindrical pericyde made up of sclerenchyma having thickness upto 50μ. The other ring of vascular bundles is very much aggregated in the pith. Vascular bundles are oval, closed and collateral. Metaxylem shows diameter of 40μ to 90μ.

T.S. of root is circular in outline surrounded by epidermal cells, length 20 to 25μ, breadth 8 to 10μ. Cortex is about 113μ in thickness and is made up of parenchymatous cells. Endodermis is present. The thickness of hollow pericycle, made up of sclerenchyma is 20 to 25μ. Vascular bundles are embeded in the pericycle. The average diameter of metaxylem is 105μ and that of protoxylem

is 30μ. Idioblasts containing ascicular crystals, length 45μ are distributed in the cortex and pith.

T.S. through the cortical region of the yam shows that tissue is differentiated into epidermis, cork layer, endodermis and ground tissue. The epidermal cell, length 15 to 30μ, breadth 10 to 15μ are compactly arranged. The cork (150μ) consists of numerous layers of thin walled flattened cells, length 25 to 55μ, breadth 15 to 25μ. The length of ascicular crystals in idioblasts, distributed in the cortex is 45 to 50μ.

The upper epidermis of leaf in T.S. consists of cells length 25 to 30μ breadth 15 to 25μ. Stomata are absent on the upper surface of the leaf but they are present on the lower surface. In T.S. it seems that midrib projects much away from the surface of lamina. the epidermis in the midrib region forms a wavy outline due to unequal distribution of cortical cells. The vascular bundle is surrounded by a zone of sclerenchyma having a thickness, 25 to 75μ. The lamina is about 165 to 225μ in thickness. It is with a single layer of pallisade cells length 50 to 65μ breadth 25 to 30μ and spongy tissue of 3 to 6 layers of cell having diameter 20 to 35μ . Lower epidermis is made up of cells length 20 to 30μ breadth 18 to 20μ The margin of the leaf part below the midrib is widely sinuate. Stomata are generally of paracytic type. Multicellular hairs having the length 100μ to 175μ are distributed mainly in the lower surface of midrib region. Idioblast cells containing ascicular crystal length 20μ are present in the lamina and midrib region.

In T.S. the outline of the basal region of the petiole is pentagonal in shape with sharp pointed projection of tissue at each angle. An average of nine vascular bundles are arranged in a circle out of which five to fine are smaller in size. Hairs are absent.

D. prazeri :The T.S. of stem is more or less circular in outline, surrounded by cuticularised epidermal cell, compactly arranged, rectangular having length 10-15μ, breadth 13μ. Epidermal hairs are absent.Cortex is made up of 5 to 10 layers of parenchymatous cell containing chloroplasts having diameter 12 to 15μ. Solid pith is made up of parenchymatous cells having diameter 45 to 60μ.

Vascular bundles are arranged in two rings. One ring of vascular bundles is apposed to the subcertical sclerenchymatous band of pericele having thickness 45 to 55μ to form a hollow cylinder. The other ring of vascular bundles are much bigger in size as compared to those apposed to the pericycle and the vascular bundles are situated more closely towards the pith. Vascular bundles are more or less oval, closed and collateral. Metaxylem in the vascular bundles away from the pericycle is much bigger in diameter (75 to 80μ) than those (20 to 25μ) apposed to the pericycle.

T.S. of root is circular in outline and is surrounded by epidermal cells length 35 to 40μ ; breadth 20 t0 2μ . There is a broad cortex (300μ) made up of parenchymatous cells. Endodermis is present. The thickness of pericycle, made up of sclerenchyma is 30 to 35μ. The vascular bundles are embeded in the pericycle. The metaxylem ranges from 45μ to 55μ. Idioblasts containing ascicular crystals having average length 50μ are distributed in the cortex.

T.S. through the cortical region of the yam shows that tissue is differentiated into epidermis, cork layer, endodermis and ground tissue. The epidermal cell length 20 to 25μ breadth 5 to 10μ are compactly arranged. The cork (100μ) consists of numerous layers of thin walled flattened cells length 20 to 45μ, breadth 10 to 15μ. The length of ascicular crystals in idioblasts distributed in cortex is 25 to 35μ.

The upper epidermis of leaf in T.S. consists of cells length 35 to 60μ breadth 30 to 50μ stomata are absent as the upper surface but they are present on the lower surface. In T.S. the midrib portion project slightly to make boat shaped outline. The vascular bundle is surrounded by a zone of sclerenchyma, 35 to 60μ in thickness. The lamina is about 200 to 250μ in thickness. It is with more or less single layer of pallisade cells length 45 to 75μ breadth 25 to 35μ. The spongy tissue is made up of 3 to 4 layer of cells having diameter 25 to 30μ. Stomata are paracytic. Hair is absent. Idioblast cells containing ascicular crystals length 25 to 35μ are distributed in the tissue of lamina.

In T.S. the outline of the basal region of the petiole is cordate in shape with a sharp knotch. An average of five vascular bundles of unequal size are distributed in a circle. Hairs are absent.

Table 8 : Some organoleptic observations on yams of different species of *Dioscorea* available in Sikkim, Darjeeling Himalayas.

Species	Feeling to touch		Taste	Odour
	Outer surface	Inner part		
D. alata	Rough	Slippery	Tasteless	No characteristic smell
D.kamoonensis	Smooth	Slippery	Tasteless	No characteristic smell
D. arachinda	Smooth	Nearly slippery	Bitter	Obnoxious smell
D. sikkimensis	Nearly smooth	Slippery	Tasteless	No characteristic smell
D. bulbifera	Smooth	Slippery	Tasteless	No characteristic smell
D.esculenta	Smooth	Slippery	Sweet	Slightly pleasant smell
D. sativum	Nearly rough	Slippery	Bitter	Obnoxious smell
D. prazeri	Nearly rough	Nearly slippery	Bitter	Slightly repulsive smell.

Table – 9 : Organoleptic observation on different species of *Dioscorea* available in Sikkim and Darjeeling Himalayas :

Species	Shape of different plant parts			Colour of different plant parts			Markings on different plant parts	
	Rhizome	Stem slightly compressed cylindrical	Leaf/ leaflet	Rhizome Outer surface	Stem	Leaf	Rhizome	Stem
D. alata	Cylindrical	Acutely augled	Leaflet ovale	Light brown	Redish green	Green	Vertical scars	No marking on angled stem
D.kamoonensis	Oblong	Cylindrical	Leaf broadly ovale	Brown	Green	Green	No marking	Marking of prickles
D. arachidna	Round	Cylindrical	Leaflet ovale	Brown	Light green	Light green	Small depressed scars	Spinus marking
D. sikkimensis	Oblong	Acutely angled with 6 wings	Leaf subhastately or deeply cordate	Brown	Greenish red	Green	Small scars	Ridged marking
D. bulbifera	Cylindrical broad at the middle	Cylindrical	Deeply leaf cordate	Yellowish brown	Light green	Green	Small scars	Ridged marking
D. esculenta	Oblong	Cylindrical	Leaf cordate	Light brown	Green	Green	No marking	Marking of prickles
D. sativum	Round	Nearly cylindrical	Leaf broadly ovale	Brownish black	Light green	Green	No marking	Marking of membrane line structure
D. prazeri	Cylindrical branched	Cylindrical	Leaf ling cordate	Brownish black	Green	Deep green	No marking	No marking

Table – 10 : Microscopic observation on leaf of different species of *Dioscorea* available in Darjeeling and Sikkim Himalayas :

Species	Epidermal cell (μ)	Type of stomata	Characteristic tissue feature of midrib	Shape at the basal region of petiole	Pallisad cell Length x Breadth (μ)	Diameterof spongy cell (μ)
D. alata	(35-45) x (25-35)	Anomocytic	Projects slight on the underside with an entirely and semi circular outline	Dome shaped	(60-75) x (25-30)	25-35
D. kamoonensis	(45-75) x (40-450)	Paracytic	Projects much on the underside with an entirely circular outline	Semi circular	(55-70) x (20-30)	25-28
D. arachidna	(45-50) x (30-35)	Paracytic	Not projected on the underside	More or less circular with a portion of flat arc.	(55-75) x (45-50)	35-40
D. sikkimensis	(40-75) x (20-50)	Paracytic	Project very much on the underside to produce an entirely obovale outline.	Dome shaped	(55-70) x (20-30)	25-35
D. bulbifera	(30-50) x (20-35)	Paracytic	Protects on the underside with deeply knotched outer surface.	Pentagonal	(40-60) x (25-35)	25-45
D. esculenta	(35-70) x (25-45)	Mixtureof paracytic and amenocytic	Projects very much on the underside to form entire oblong outline.	Circular	(100-110) x (35-55)	20-40
D. sativum	(25-30) x (15-25)	Paracytic	Projects very much on the underside with wavy in outline	Pentagonal	(50-65) x (25-30)	20-35
D. prazeri	(35-60) x (30-50)	Paracytic	Project slightly to make boat shaped outline.	Cordate with a sharp knotch	(45-75) x(25-35)	25-30

Table – 11 : Some microscopic observations on stem of different species of *Dioscorea* available in Darjeeling-Sikkim Himalayas :

Name of the species	Shape in T.S.	Epidermal cell length x Breadth (μ)	Cell layers of Cortex	Epiderma l hair	Arrangement of vascular bundle	Tchickness of pericycle layer (μ)	Diameter of metaxylern (μ)
D. alata	Elliptric	(20-25) x (15-17.5)	4-5	Absent	Vascular bundles arranged in one row embeded in pericycle	100-130	50-80
D. kamoonensis	Circular	(40-45) x (30-35)	6-8	Present	Vascular bundles arranged in two rings. One embeded in pericycle other move towards the pith	60-75	100-110
D. arachidna	Circular	(30-35) x (20-22)	5-6	Present	Vascular bundle arranged in two rings, one embeded in the pericycle, other distributed in pith.	140-150	60-100
D. sikkimensis	More or less triangular with a curved base	(25-28) x (13-15)	3-4	Absent	Vascular bundle arranged in two rings, one apposed tot he pericycle, the other more towards pith	20-140	55-110
D. bilbifera	Pentagonal with curved arms	(12-15) x (6-10)	3-5	Present	Vascular bundle arranged in two rings, one apposed to pericycle, the other more towards pith.	30-45	50-90
D. esculenta	Circular	(20-25) x (18-20)	6-9	Present	Vascular bundle arranged in two rings, a ring of small vascular bundle embeded in pericycle , the other larger oes are much away from the pericycle.	25-30	100-170
D. sativum	Polygonal with curved sides	(15-20) x (10-18)	5-6	Present	Vascular bundle arranged in two rings, one apposed to pericycle, others are aggregated in the pith.	40-50	40-90
D. prazeri	Circular	(15-20) x (10-15)	5-10	Absent	Vascular bundle arranged in two rings, one apposed to pericycle, other very much bigger, more towards pith.	45-55	20-80

Table – 12 : Some microscopic observations on root of different species of *Dioscorea* available in Darjeeling and Sikkim Himalayas.

Species	Epidermal cell (μ)	Thickness of Cortex (μ)	Thickness of pericycle layer (μ)	Arrangement of vascular bundle	Distribution of idioblasts
D. alata	(50-55) x (25-30)	325	90-100	In two ring one embeded in pericycle , the other much away from it.	Corte and Pith
D.kamoonensis	(20-35) x (12-15)	240	65-70	Vascular bundles are apposed to the pericyclic layer.	Cortex and pith
D. arachinda	(15-20) x (14-18)	105	180-225	Vascular bundles are embeded in the pericycle.	Pith
D. sikkimensis	(20-3) x (12-15)	230	400-425	Vascular bundles embeded in the pericycle.	Cortex and pith
D. bulbifera	(15-35) x (12-15)	260	40-45	Vascular bundles touches to the pericycle layer.	Cortex and pith
D. esculenta	(30-35) x (15-20)	145	300-310	Vascylar bundles are embeded in the pericycle.	Pith
D. sativum	(20-24) x (8-10)	113	20-25	Vascular bundles are embeded in the pericycle.	Cortex and pith
D. prazeri	(35-40) x (20-25)	300	30-35	Vascular bundles are embeded in the pericycle.	Cortex

Table 13 : Some microscopical observation on yam of different species of *Diascorea* available in Darjeeling and Sikkim, Himalayas.

Species	Epidermal cell length x Breadth (μ)	Thickness of cork layer (μ)	Distribution of idioplasts containing ascicular crystals	Length of ascicular (μ) crystals
D. alata	(25-27) x (15-17.5)	195	Ground tissue	70-75
D. kamoonensis	(35-40) x (20-25)	200	Ground tissue	50-55
D. arachidna	(15-18) x (10-12)	150	Ground tissue	40-55
D. sikkimensis	(12-15) x (8-10)	155	Ground tissue	34-40
D. bulbifera	(20-25) x (8-10)	125	Gournd tissue	45-50
D. esculenta	(20-30 x (15-18)	225	Gournd tissue	45-48
D. sativam	(15-30) x (10-15)	150	Gournd tissue	45-50
D. prazeri	(20-25) x (5-10)	100	Gournd tissue	25-35

Table 14 : Observation on quantitative microscopy in leaf of different species of *Dioscorea*

Species	Stomatal index (Lower surface of leaf)	Pallisade ratio (Upper surface of leaf)
D. alata	24.50	38
D. kamoonensis	28.66	32
D. arachidna	21.60	36
D. sikkimensis	30.00	46
D. bulbifera	27.00	48
D. esculenta	20.10	30
D. sativun	21.00	59
D. prazeri	19.00	50

Fig: 11. Yam of *D.alata.*

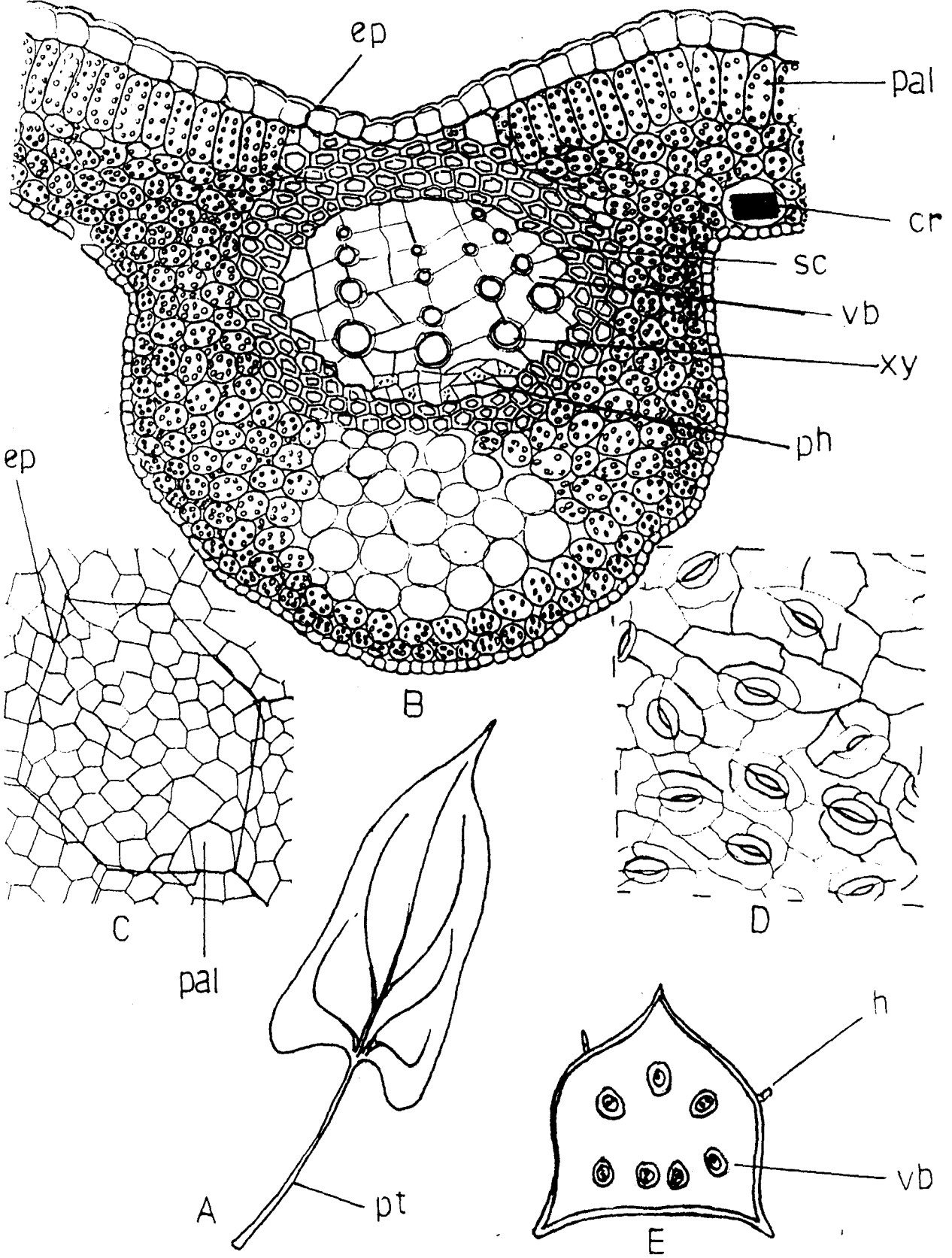

Fig : 12.*D.alata*, A.Leaf **B**. T.S.of leaf through midrib, **C**. Epidermal and pallisade cells. **D**.Epidermal clls and stomata. **E**. T.S.through the basal region of petiole (Diagrammatic). ep-epidermal cell, cr-crystals, h-hair, pal-pallisade cell, ph-phloem. pt- petiole, sc- sclerenchyma, vb-vascular bundle xy-xylem.

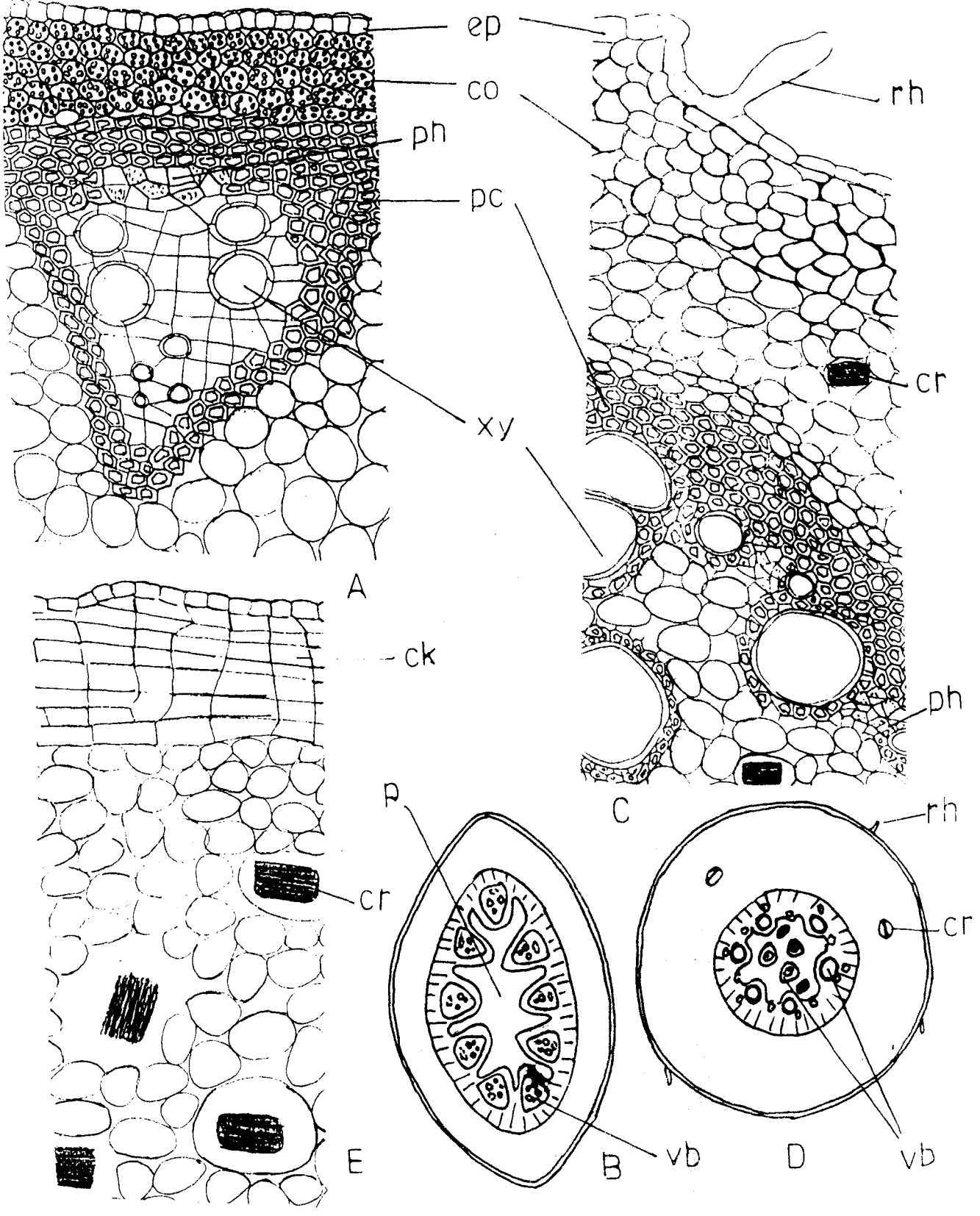

Fig : 13. ***D.alata.*** **A**. T.S.of a part of stem. **B**. T.S.of stem (Dia grammatic), **C**. T.S.of a part of root, **D**. T.S.of root (dliagrammatic) **E**. T. S.of a part of yam, ck-cork, co-cortex, cr-crystals, ep-epidermal cell, h-hair, p-pith, pc-pericycle, ph-phloem, rh-root hair; vb- vascular bundle, xy-xylem.

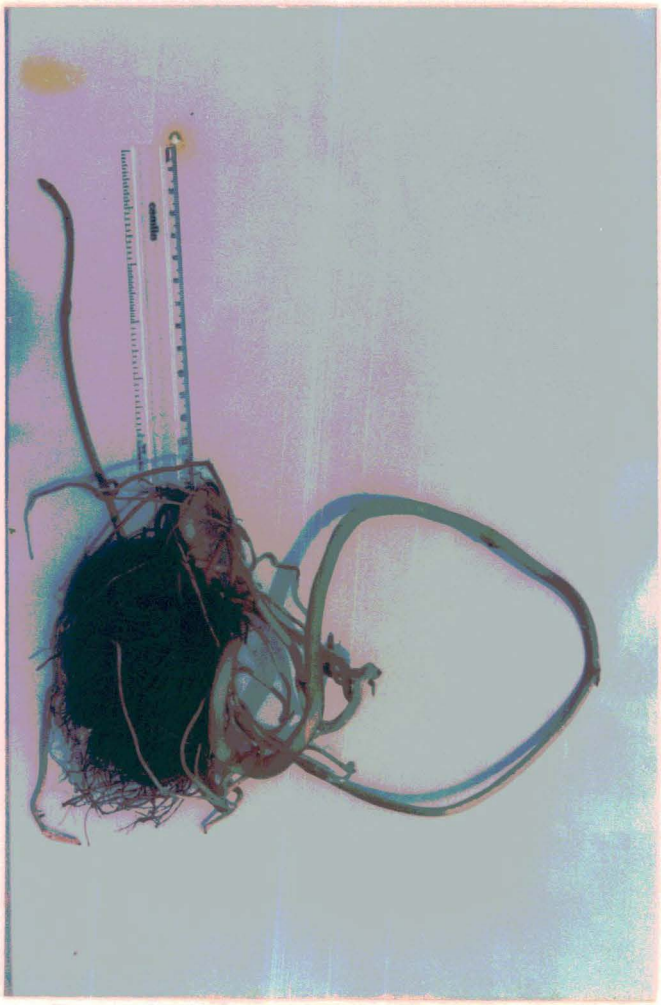

Fig: 14. Yam of *D. kamoonensis.*

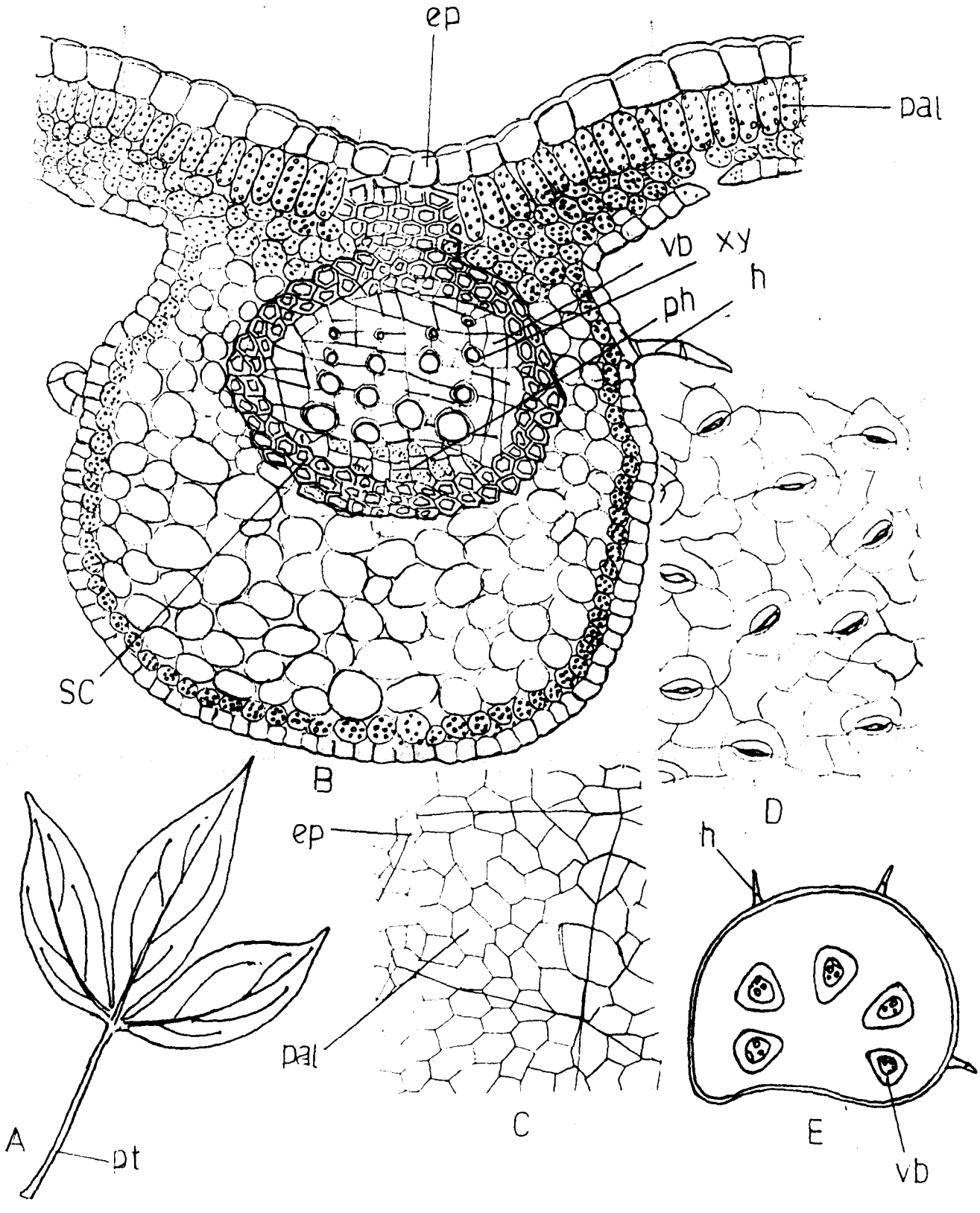

Fig : 15 ***D. kamoonensis.*** A. leaf B. T.S.of leaf through midrib C. Epidermal and pallisade cells, D.Epidermal cells and Stomata.E.T.S.through the basal region of petiole.(Diagrammatic) ep- epidermall cell, h - hair, pal - pallisade cell, Ph - Phloem. Pt - Petiole sc- Sclerenchyma. ble-Vascular bundle, Xy -Xylem.

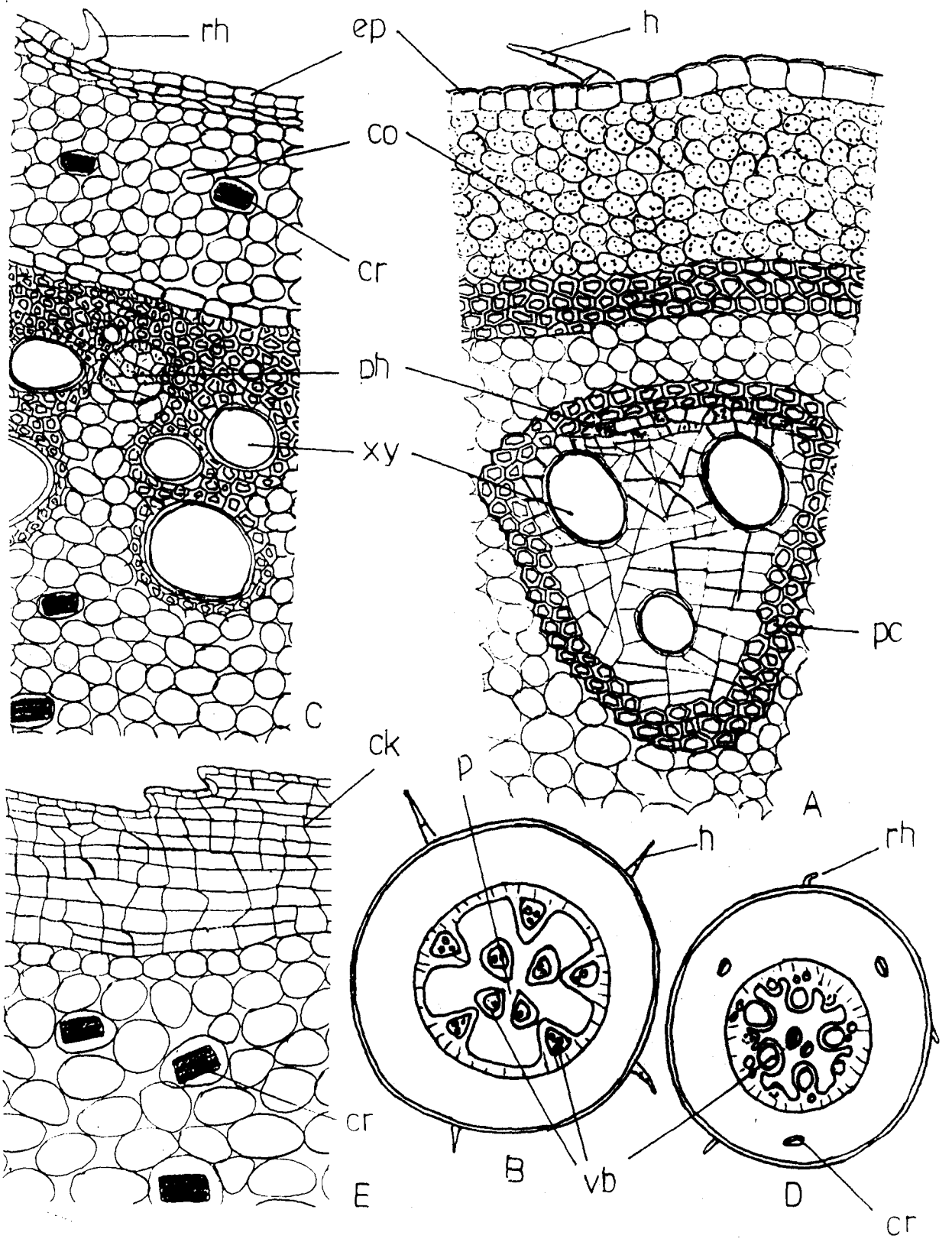

Fig: 16. ***D. kamoonensis.*** **A**. T.S.of a part of stem. **B**. T. S.of stem (Diagrammatic), **C**. T. S. of a part of root. **D**.T.S. of root (Diagrammetric). **E**. T.S.of a part of yam.ck-cork, co-cortex, cr-crystals, ep-epidermal cell, h-hair, p-pith, pc-pericyche, ph-phloem, rh-root hair, vb- vascular bundle, xy - xylem.

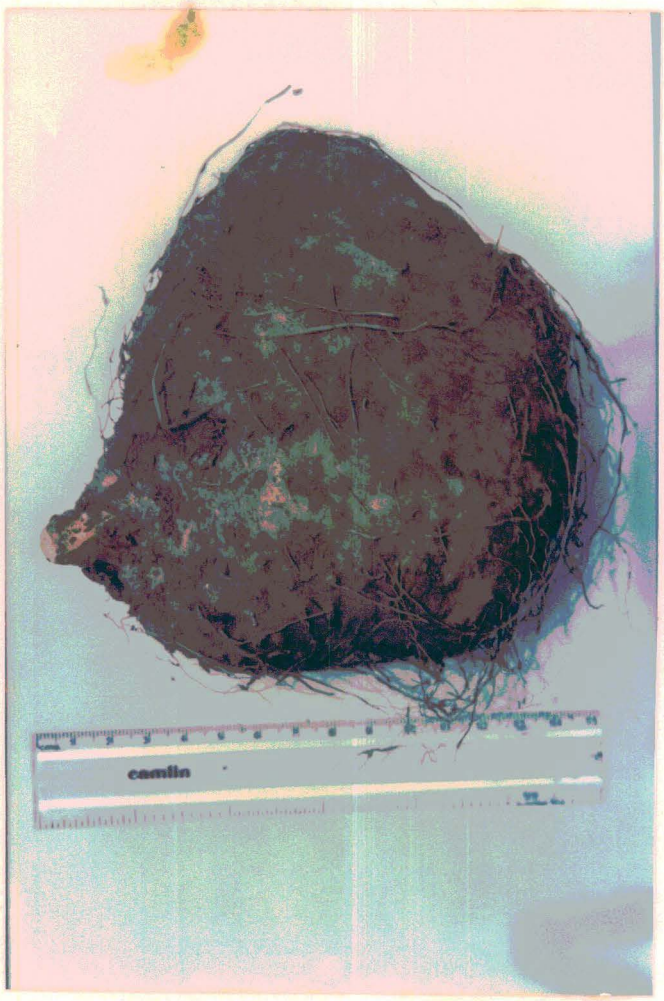

Fig : 17 Yam of *D.arachidna.*

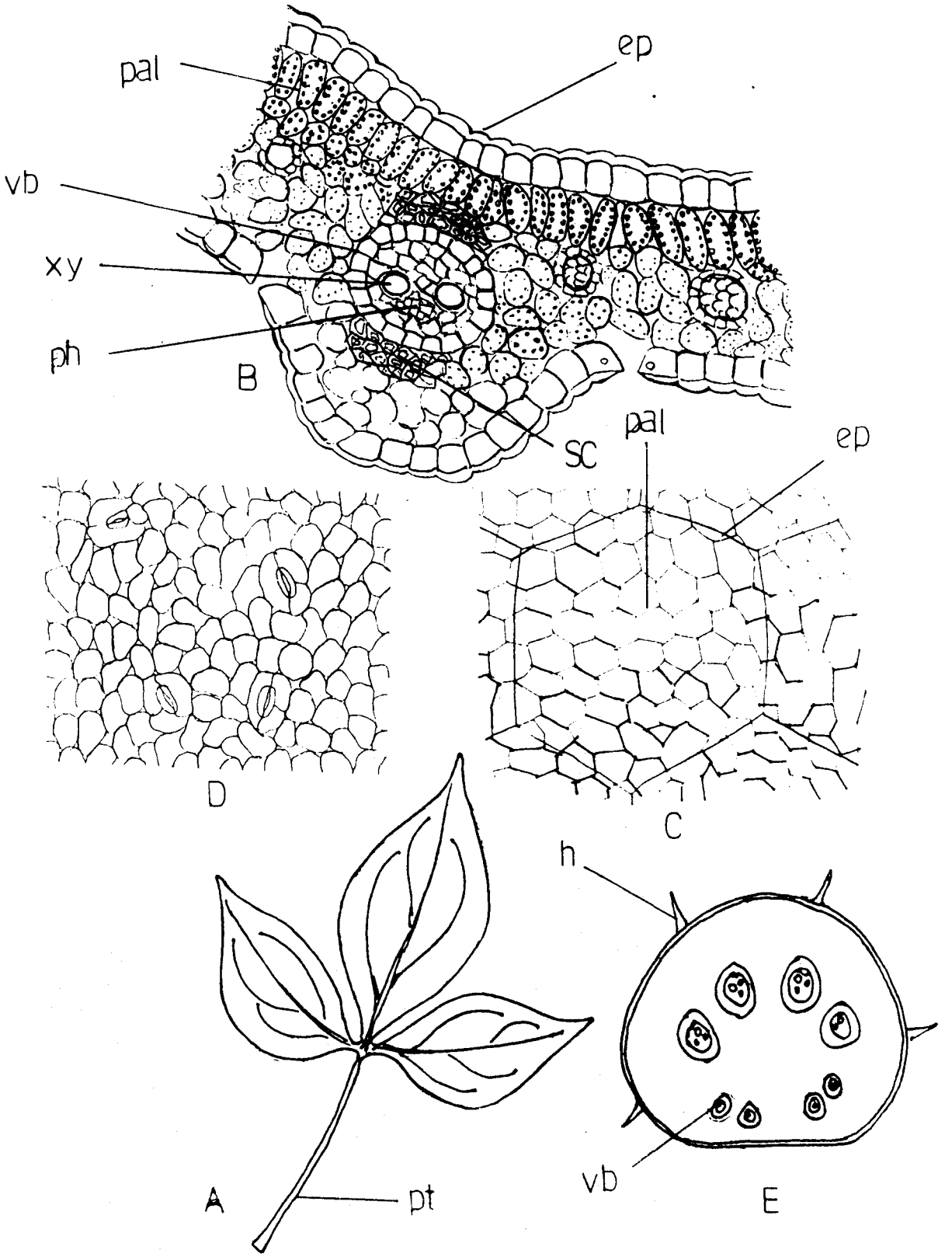

Fig: 18. ***D. arachidna,*** **A.** Leaf. **B**. T. S. of leaf through midrib. **C**. Epidermal and pallisade cells. **D**.Epidermal cells and Stomata, **E**. T. S. through basal region of petiole (Diagrammatic). eP-epidermal cell,h - hair, pal- pallisalde cell, ph - phloem, pt- petiole, sclerenchyma.vb- vascular bundle xy-xylem.

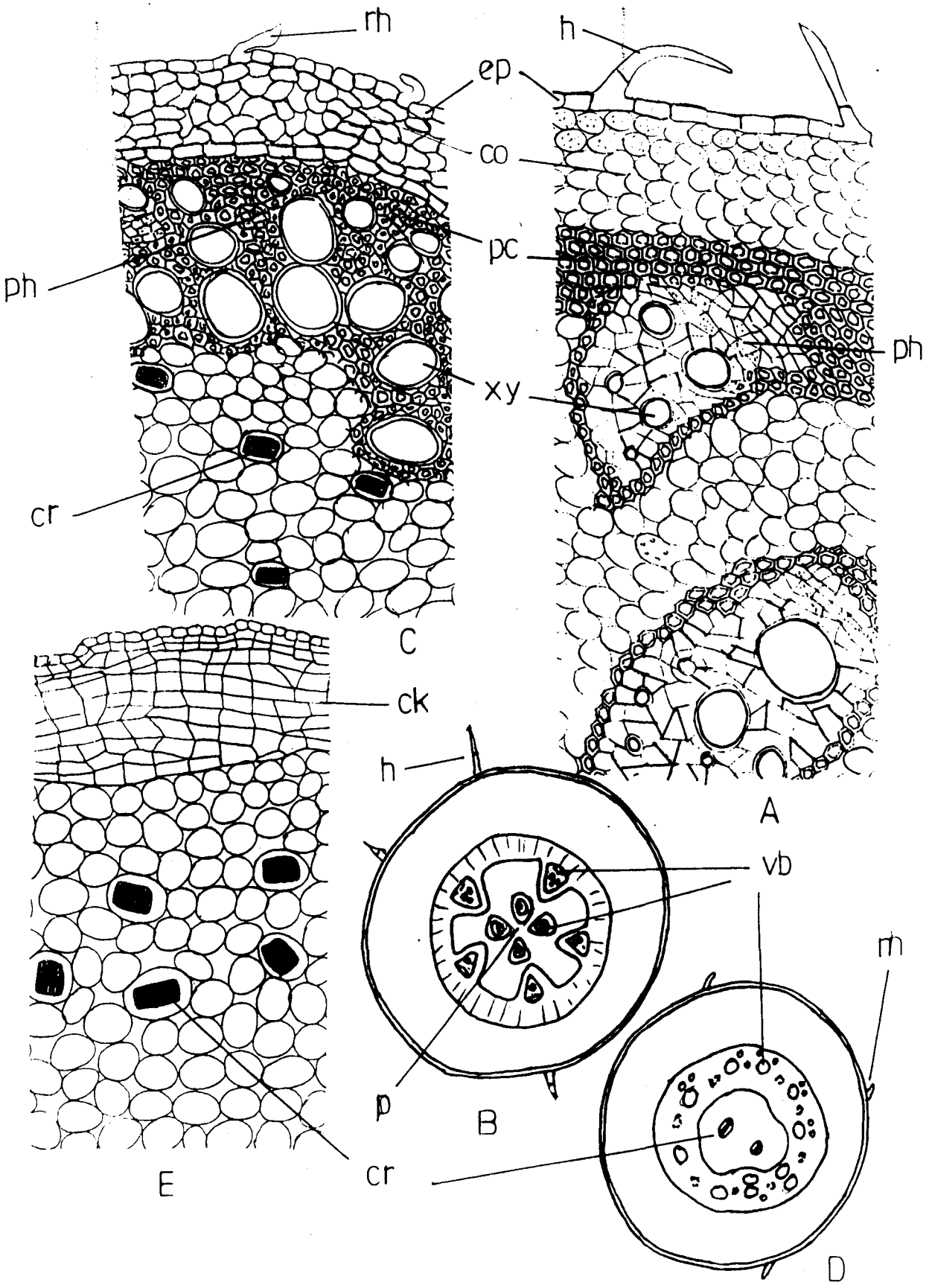

Fig: 19. ***D.arachidna*** **A**. T. S. of a part of stem **B**. T.S.of Stem (Diagrammatic) **C**. T.S. of a part of root. **D**.T.S.of root (Diagrammatic) **E**. T.S.of a part of yam. ck-cork. co-cortex.cr-crystals.ep-epidernal cells, h-hair, p-pith, pc-pericycle. ph-phloem, rh-root hair. vb-vascular bundle, xy -xylem.

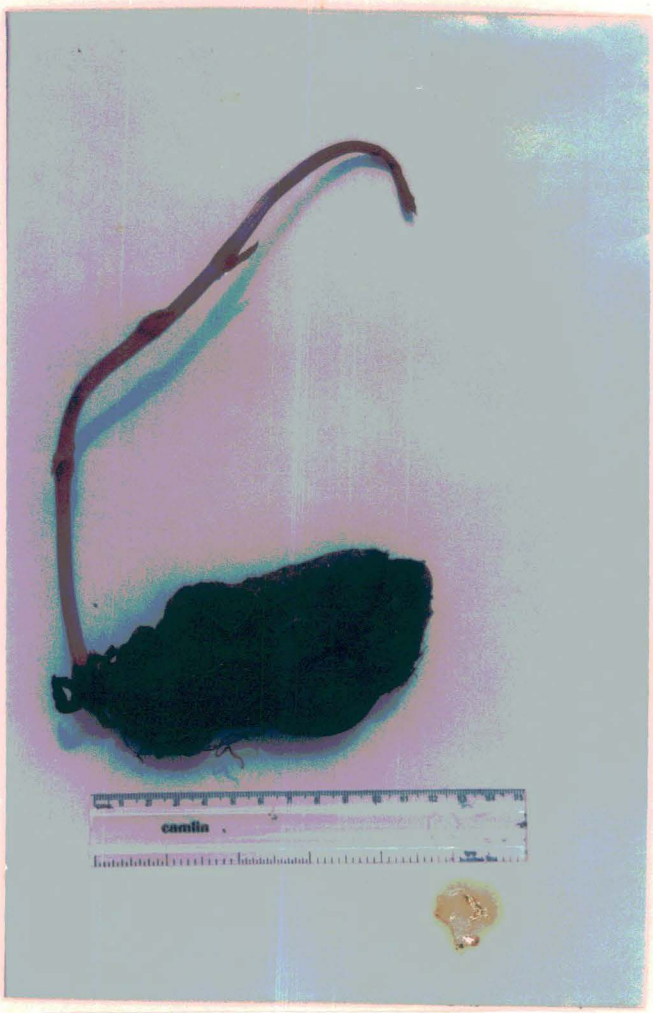

Fig : 20. yam of *D. sikkimensis.*

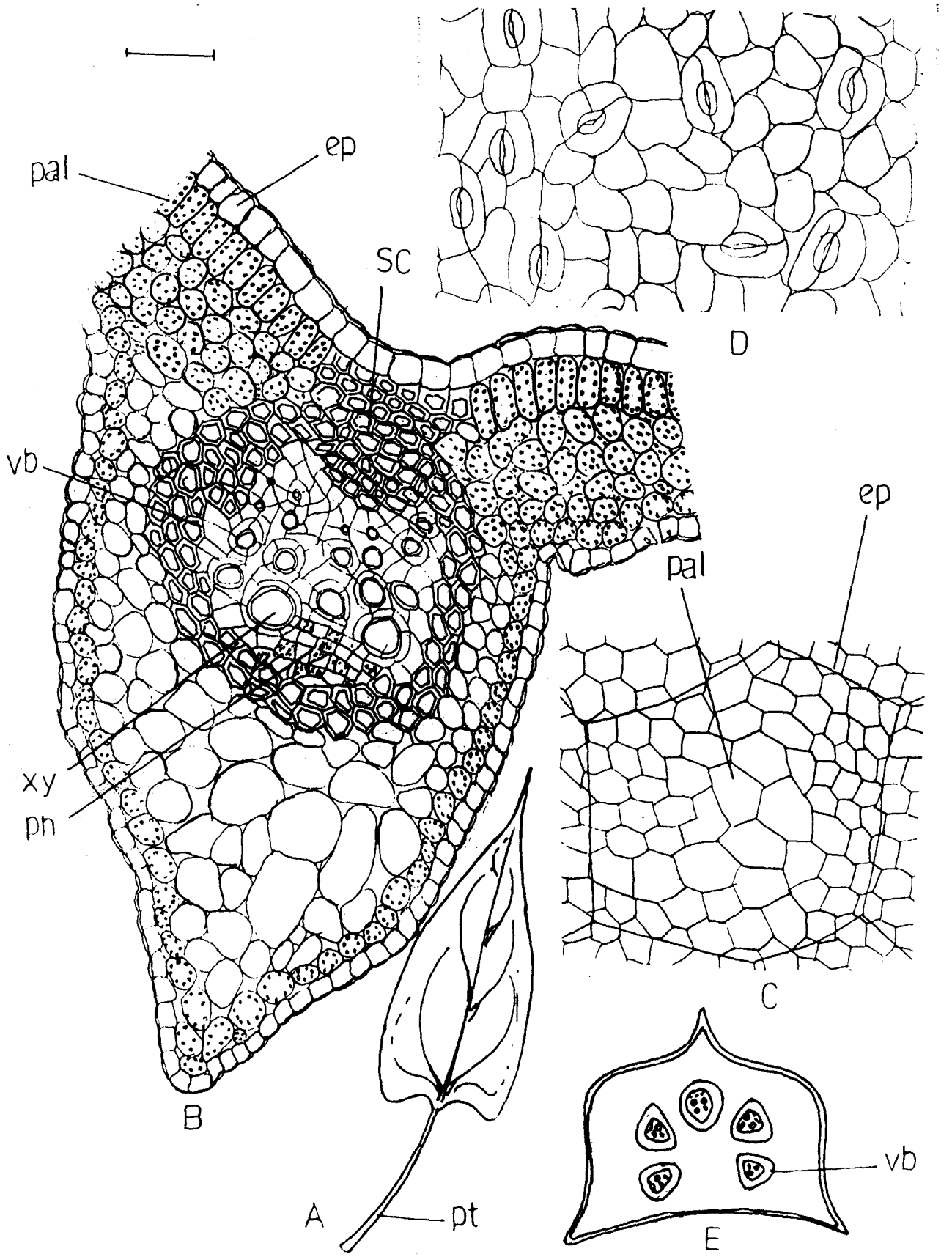

Fig :21. ***D. sikkimensis,*** **A**. Leaf **B**. T. S. of leaf through midrile, **C**. Epidermal cells. and pallisade cells. **D**. Epidermal cells and stomata. **E** T.S. through the basal region of petiole.(Diagrammatic) ep-epidermal cell, pal-pallisade cell, ph-phloem, pt- petiole sc-sclerenchyma, vb-vascular bundle xy -xylem.

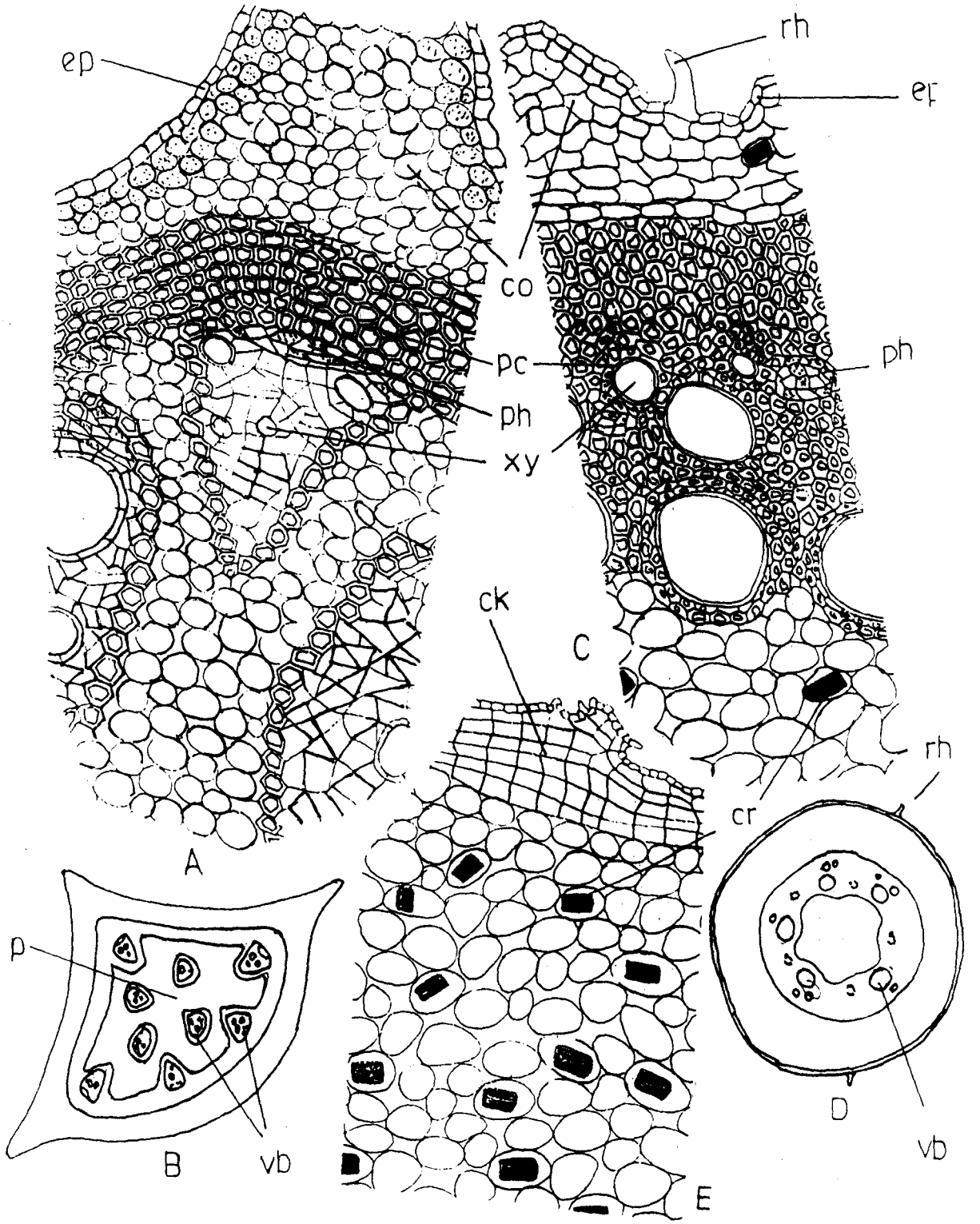

Fig: 22.*D. sikkimensis*, A. T. S. of a part of stem, **B**. T. S.of stem (Diagrammatic) **C**. T. S. of a part of root **D**. T. S.of root (Diagrammatic). **E**. T. S.of a part of yam. ck-cork.co-cortex, cr- crystals, et-epidermal cells, p-pith, pc-pericycle ph-phloem, rh-root hair, vb-vascular bundle xy-xylem.

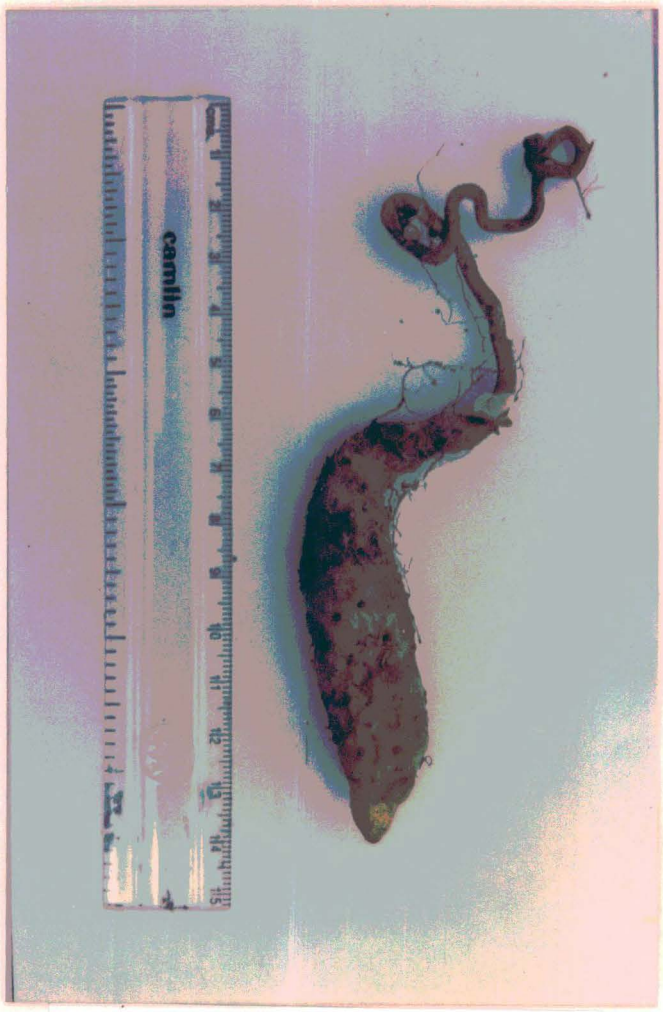

Fig: 23. yam of *D. bulbifera.*

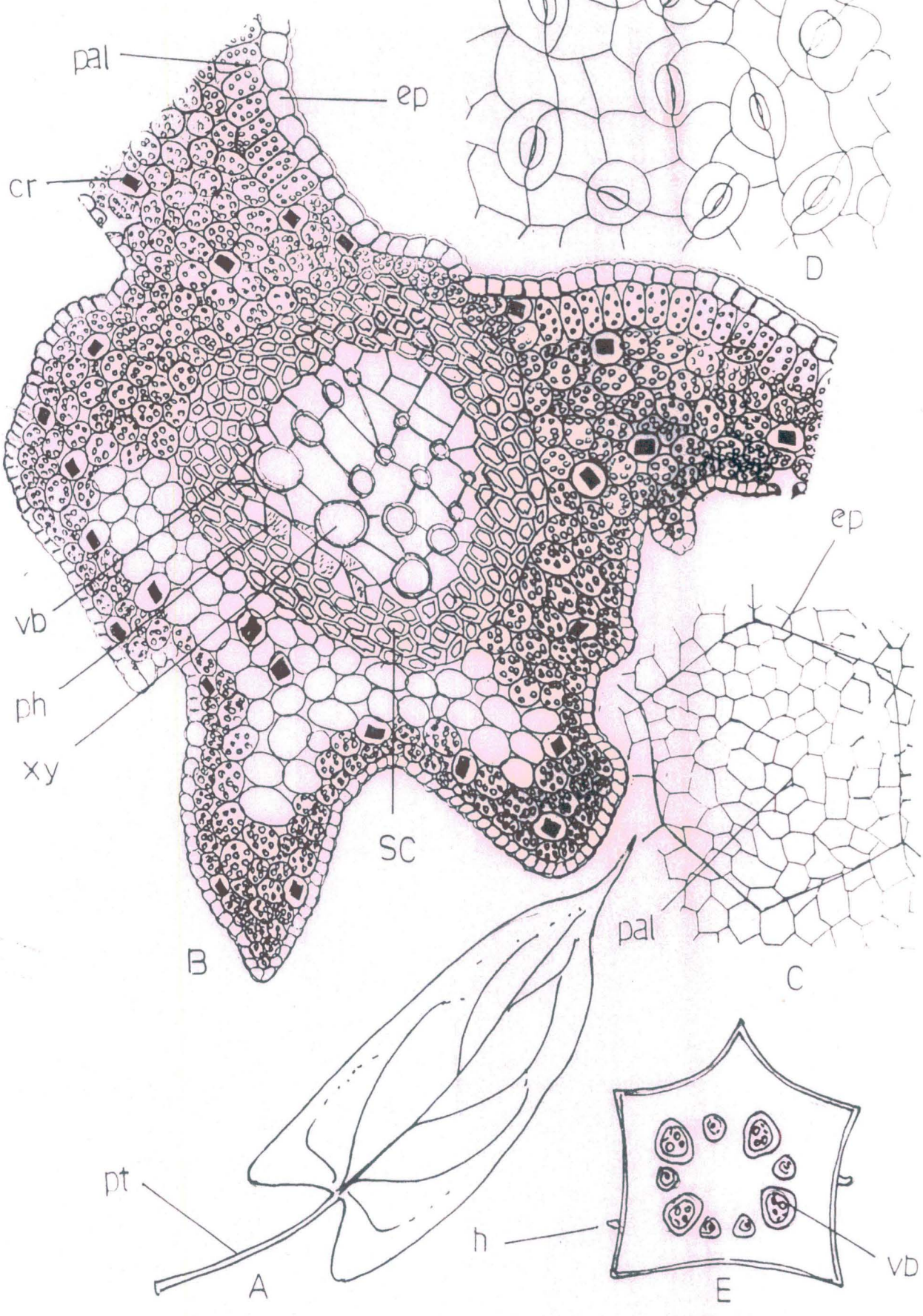

Fig: 24. ***D. bulbifera*** **A**. Leaf. **B**. T. S.of leaf through midrib **C**. Epidermal and pallisade cells.**D**.Epidermal cells and stomata. **E**. T. S.through the basal region of petiole.(Diagrammatic) ep-epidermal cell cr-crystals, h-hair, pal pallisade cell, ph-phloem. pt-petiole, sc- sclerenchyma, vb- vascular bundle, xy - xylem.

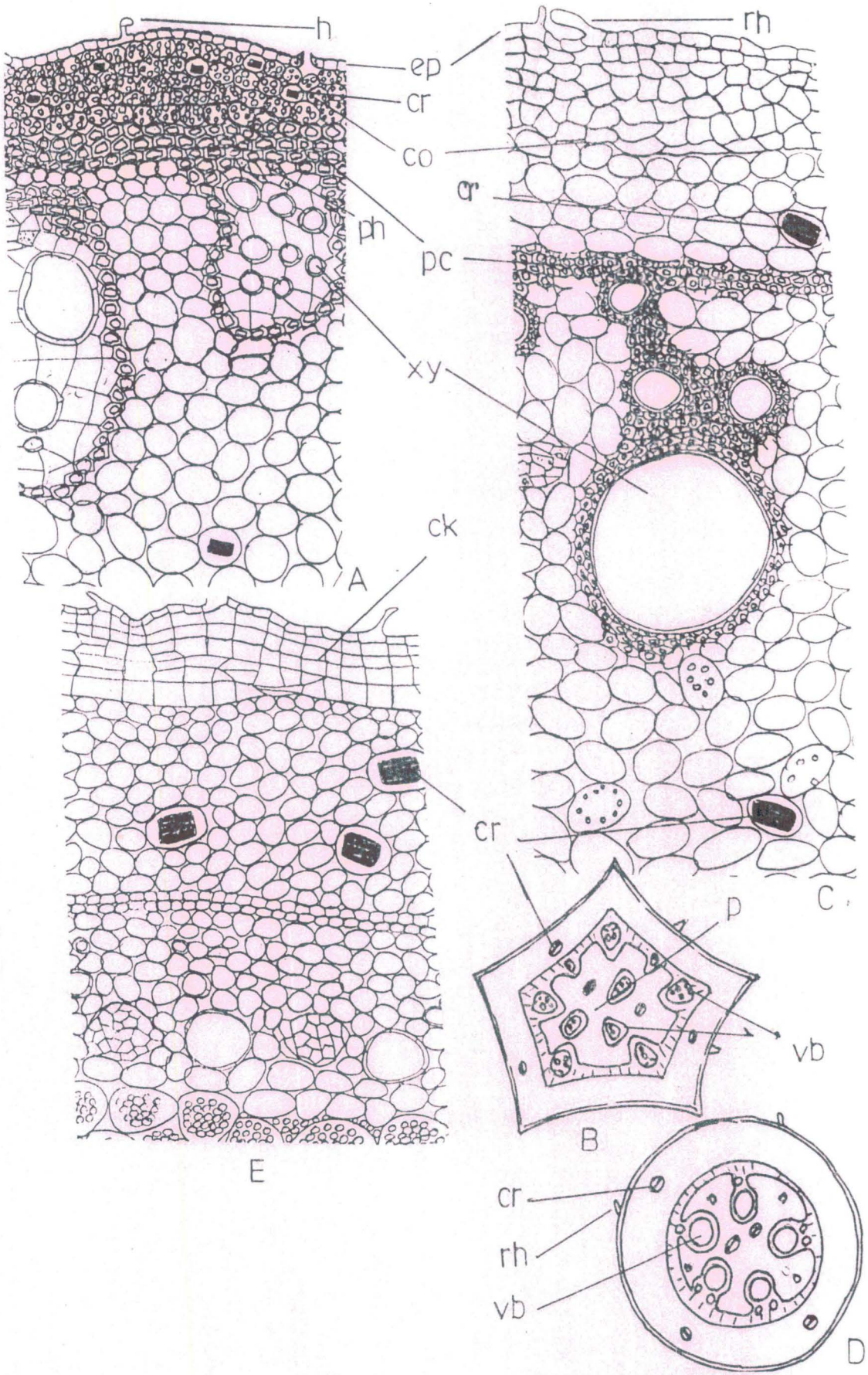

Fig: 25. ***D. bulbifera*** **A**. T. S.of a part of stem. **B**.T.S.of stem (Diagrammatic), C.T.S.of a part of root, **D**. T. S.of root (Diagrammatic) **E**.T.S.of a part of yam.ck-cork, co-cortex, cr-crystals, ep-epidermal cell, h-hair, p-pith, pc-pericycle, ph-phloem, rh-root hair, vb-vascular bundle, xy-xylem.

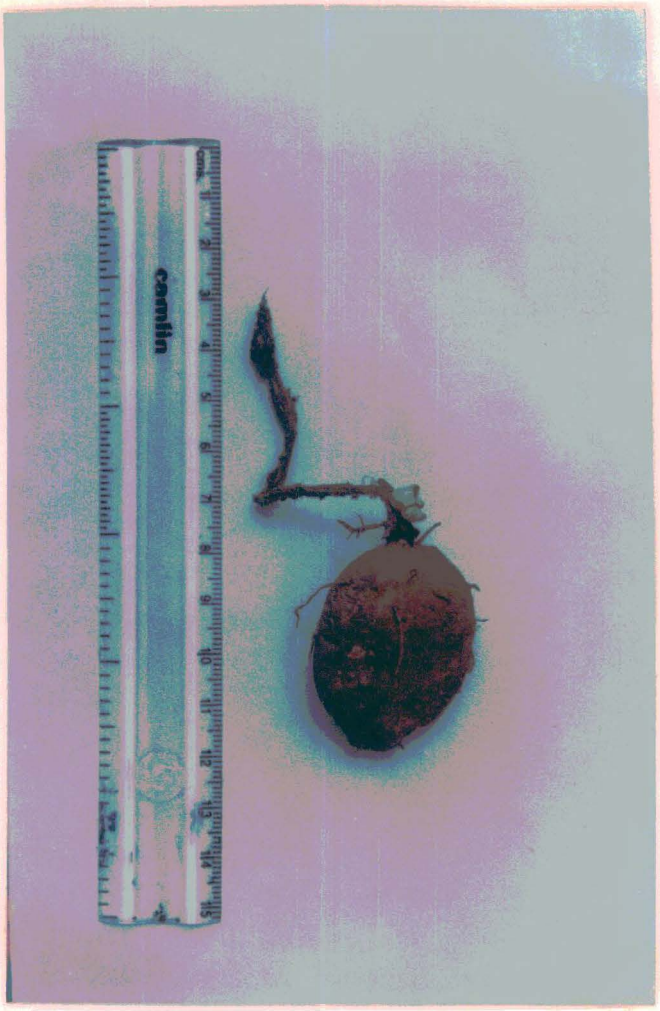

Fig: 26. Yam of *D. esculenta.*

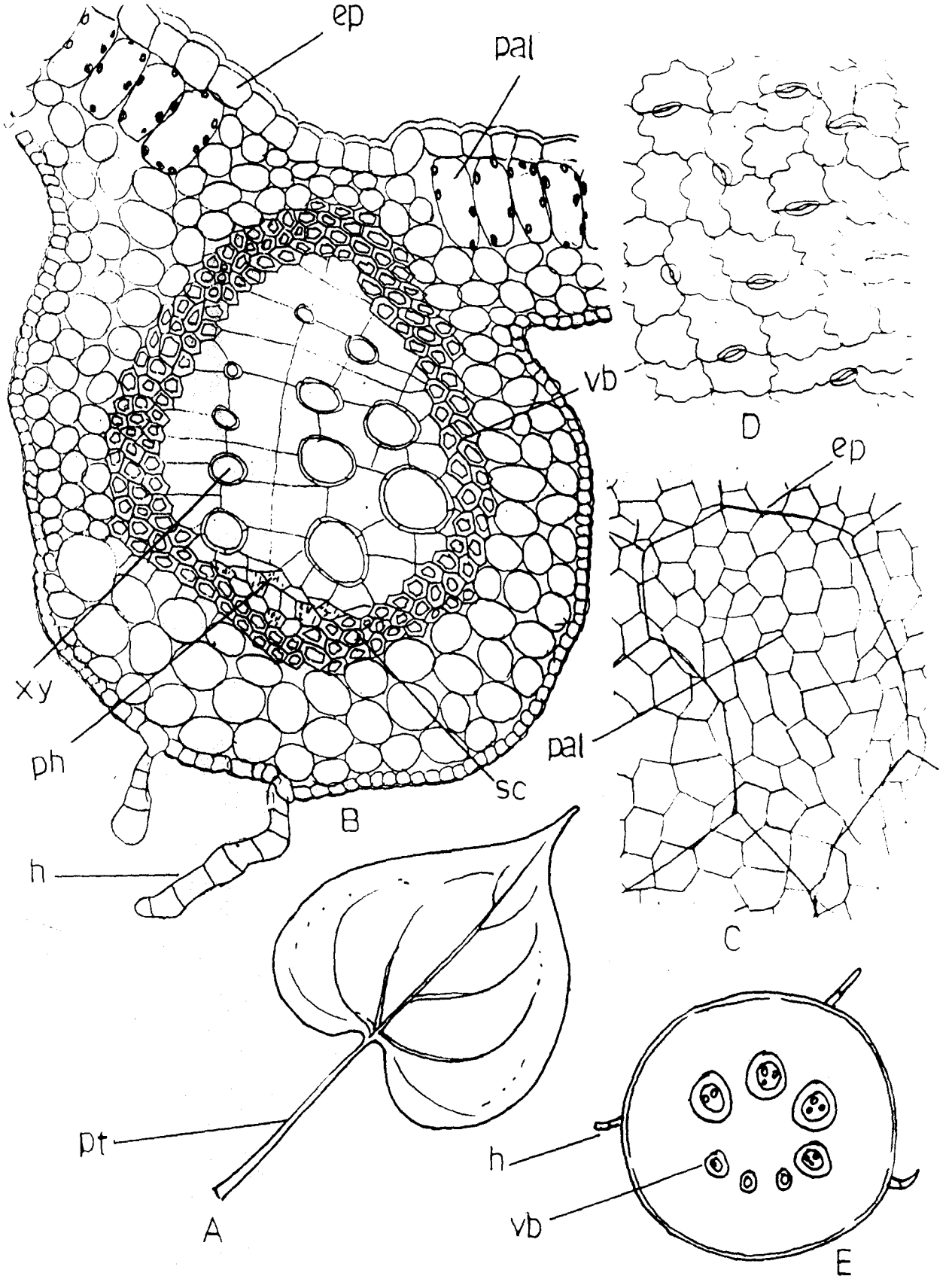

Fig: 27. ***D.esculenta,*** **A**.Leaf. **B**.T.S.of leaf through midrib.**C**.Epidermal and pallisade cells. **D**. Epidermal cells and stomata. **E**.T. S. through the basal region of petiole.(Diagrammatic).ep - epidermal cell. h-hair, pal-pallisade cell, ph-phloem, pt-petiole, sc-sclerenchyma, vb-vasecular bundle. xy-xylem.

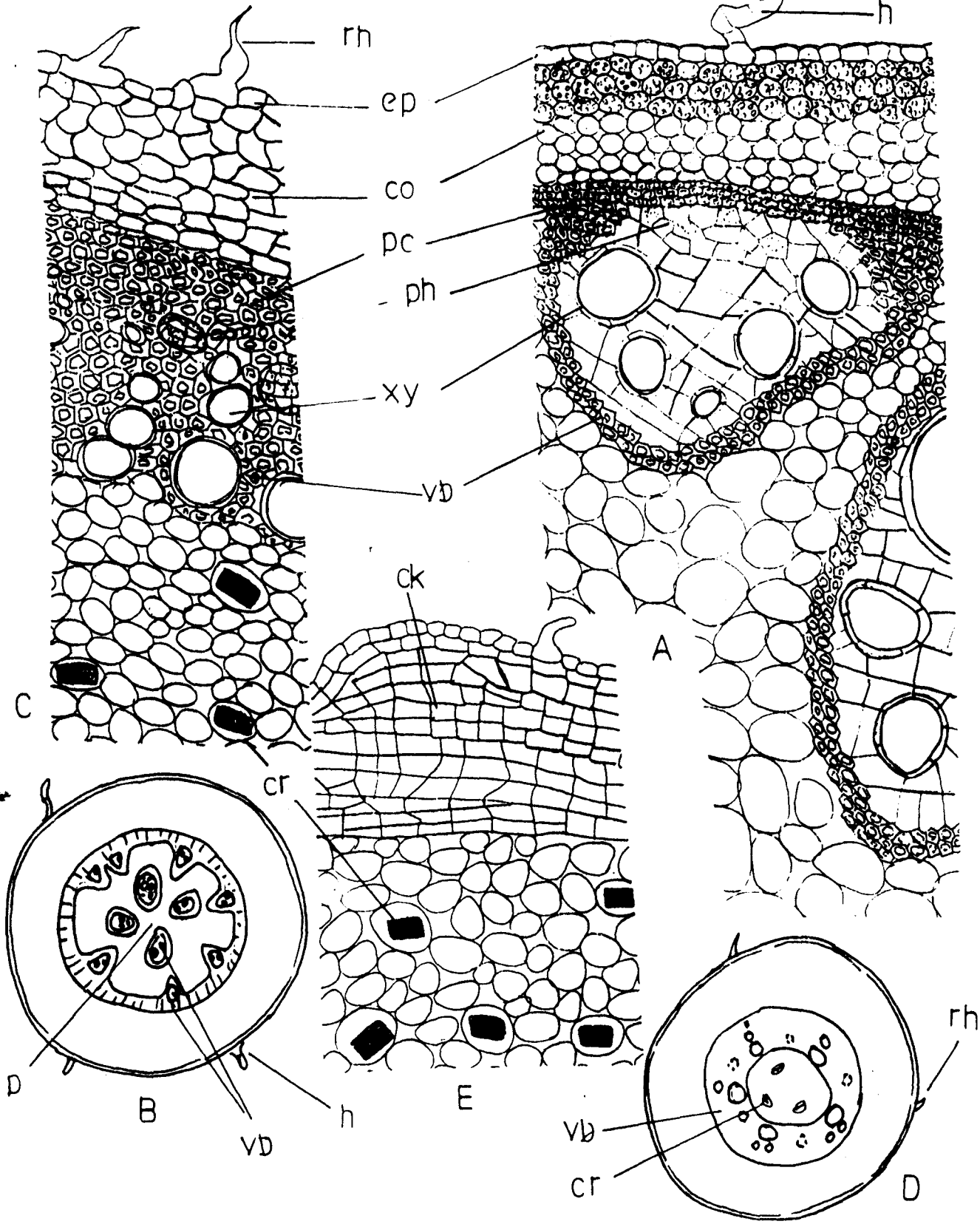

Fig: 28. ***D. esculenta*** **A**. T. S. of a part of stem. **B**. T.S. of stem (Diagrammatic) **C**.T. S of.a part of root **D**. T. S.of root (Diagrammatic) **E**.T.S.of a part of yam, ck-cork, co-cortex, cr-crystals, ep-epidermal cell, h- hair, p-pith, pc.pericycle, ph-phloem, rh-root hair, vb-vascular bundle, xy - xylem.

Fig: 29. Yam of *D. sativum.*

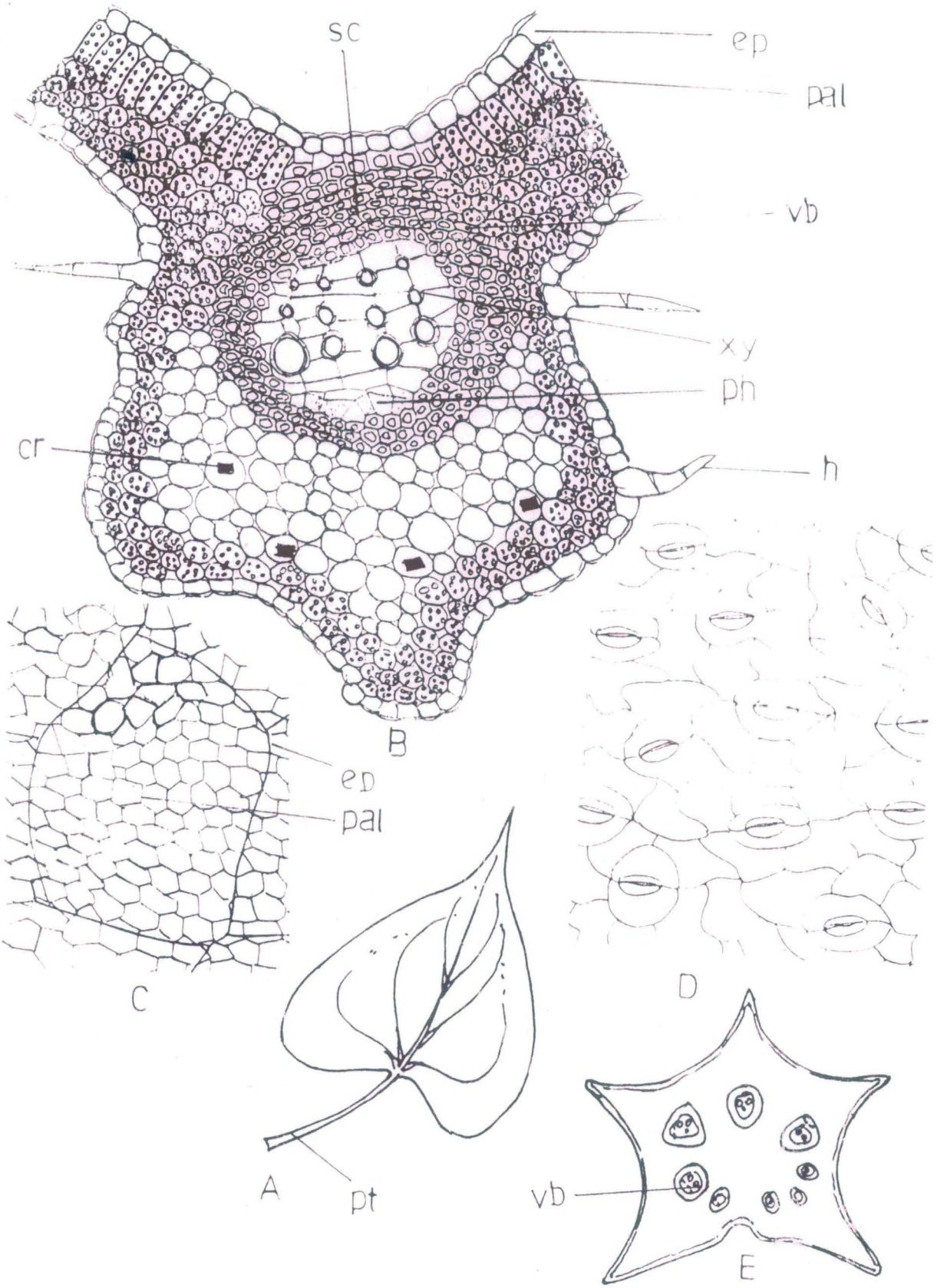

Fig: 30. ***D. sativum*** **A.** Leaf **B**. T. S. of leaf. through midrib. **C**. Epidermal and pallisade cells **D**. Epidermal cells and stomata **E**. T. S through the basal region of petiole (Diagrammatic) ep-epidermal cell, h-hair pal - pallisade cell, ph-phloem pt-petiole sc-sclerenchyma, vb-vascular bundle xy-xylem

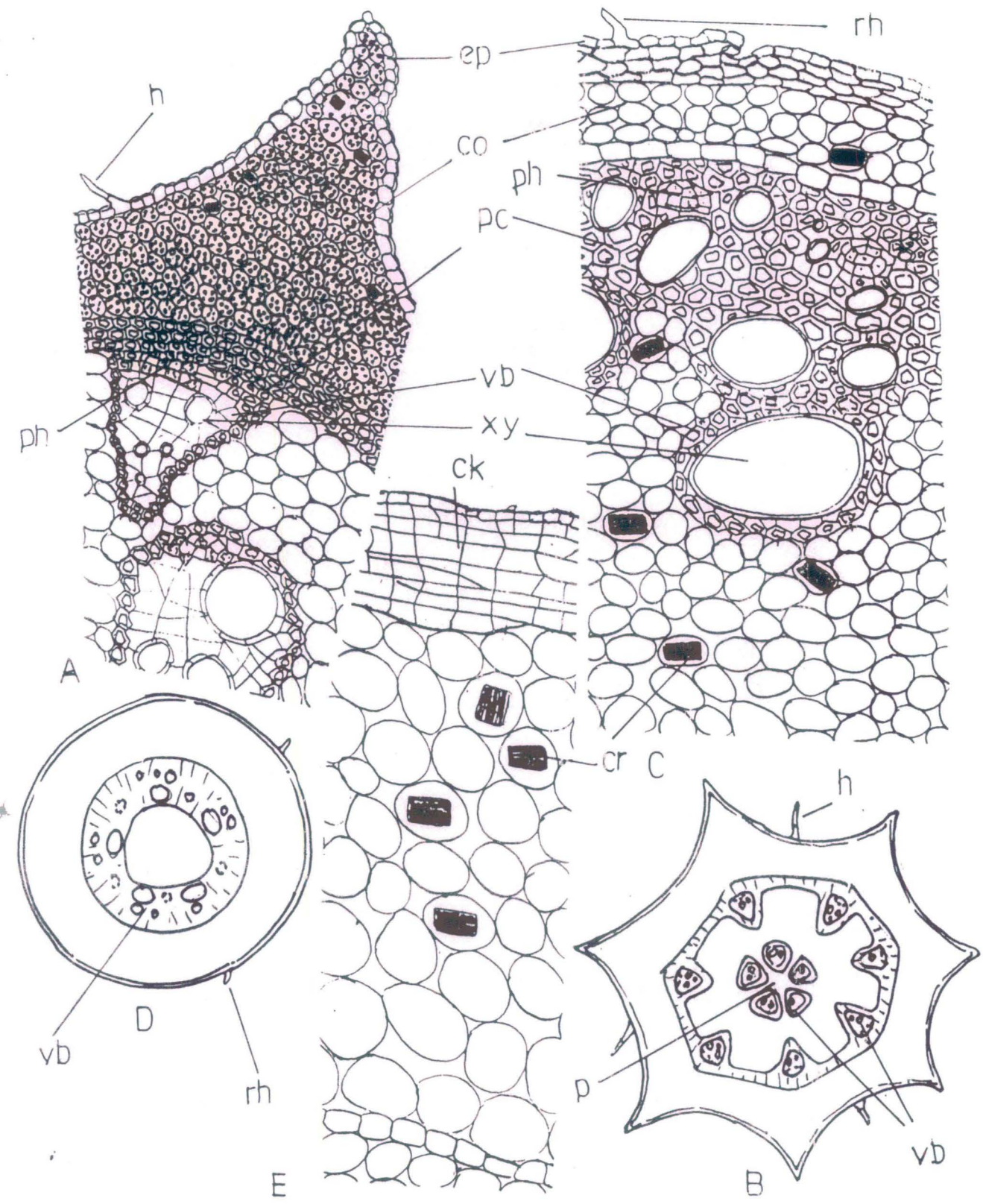

Fig : 31. ***D. sativum*** **A**. T. S.of a part of stem, **B**. T.S. of stem (Diagrammetic) **C**. T.S. of a part of root. **D**. T.S. of root (Diagrammetic) **E**. T.S.of cr-crystals, ep-epidermal cells, h-hair, p-pith rh-root hair, vb - vascular bundle, xy-xylem.

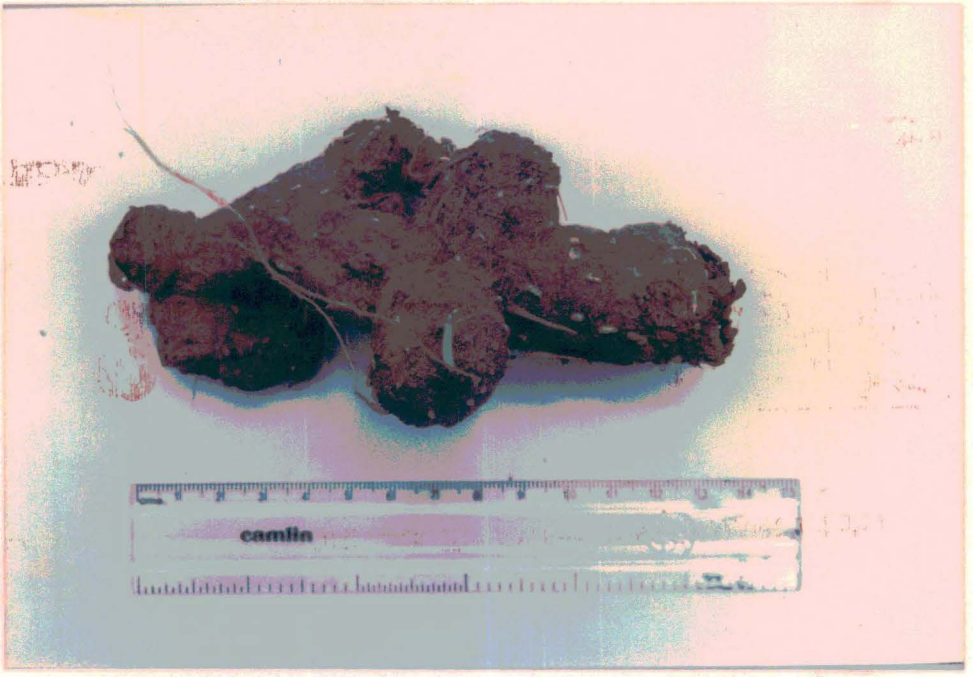

Fig: 32. Yam of *D. prazeri.*

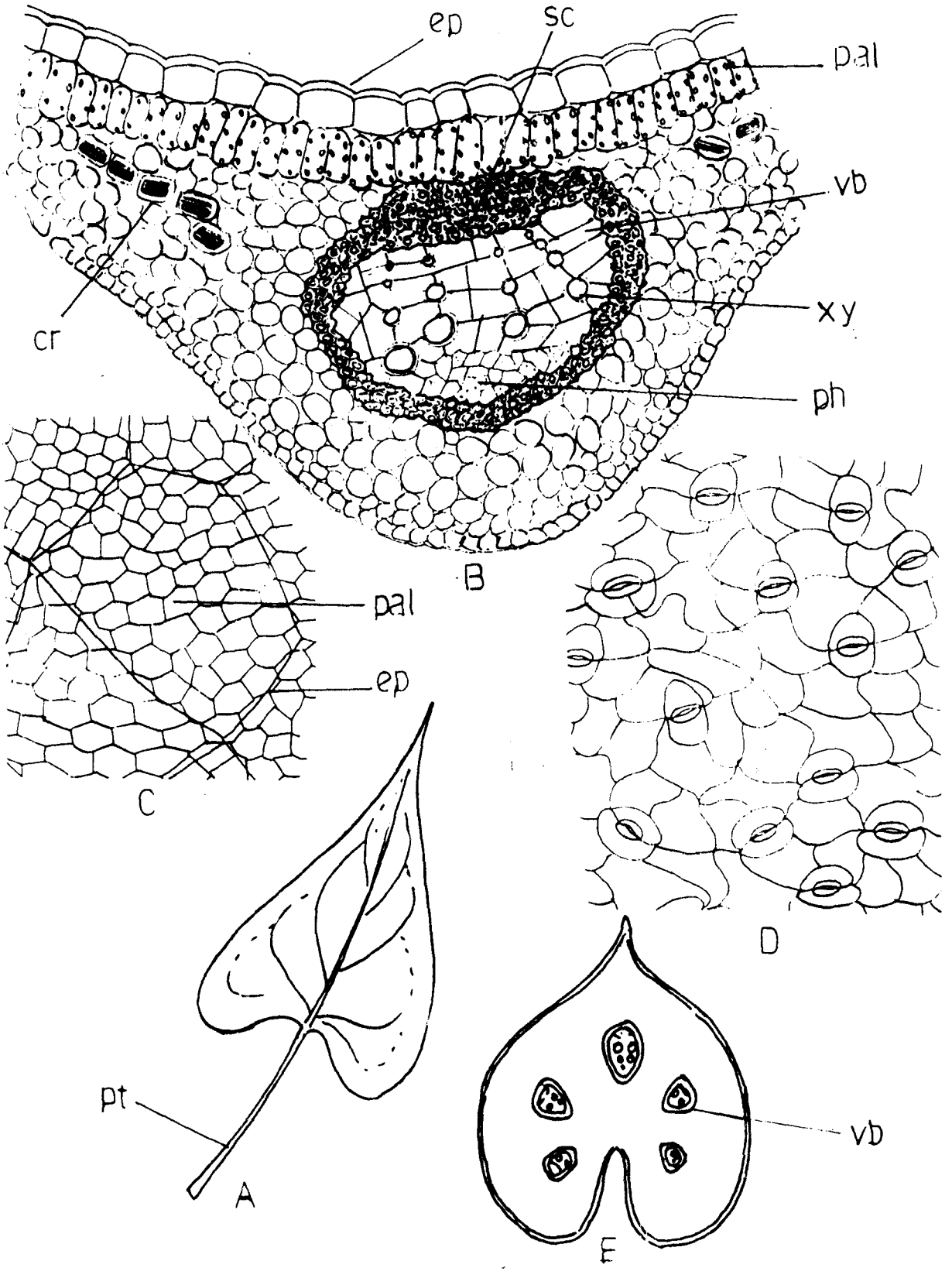

Fig : 33. ***D. prazeri*** **A** Leaf, **B**.T. S.of leaf through midrile. **C**. Epidermal cells and pallisade cells. **D**. Epidermal cells and stomata **E**. T.S. through the basal region of petiole (Diagrammatic).ep-epidermal cell, pal-pallisade cell, ph-phloem, pt-petiole, sc-sclerenchyma, vb-vascular bundle, xy-xylem.

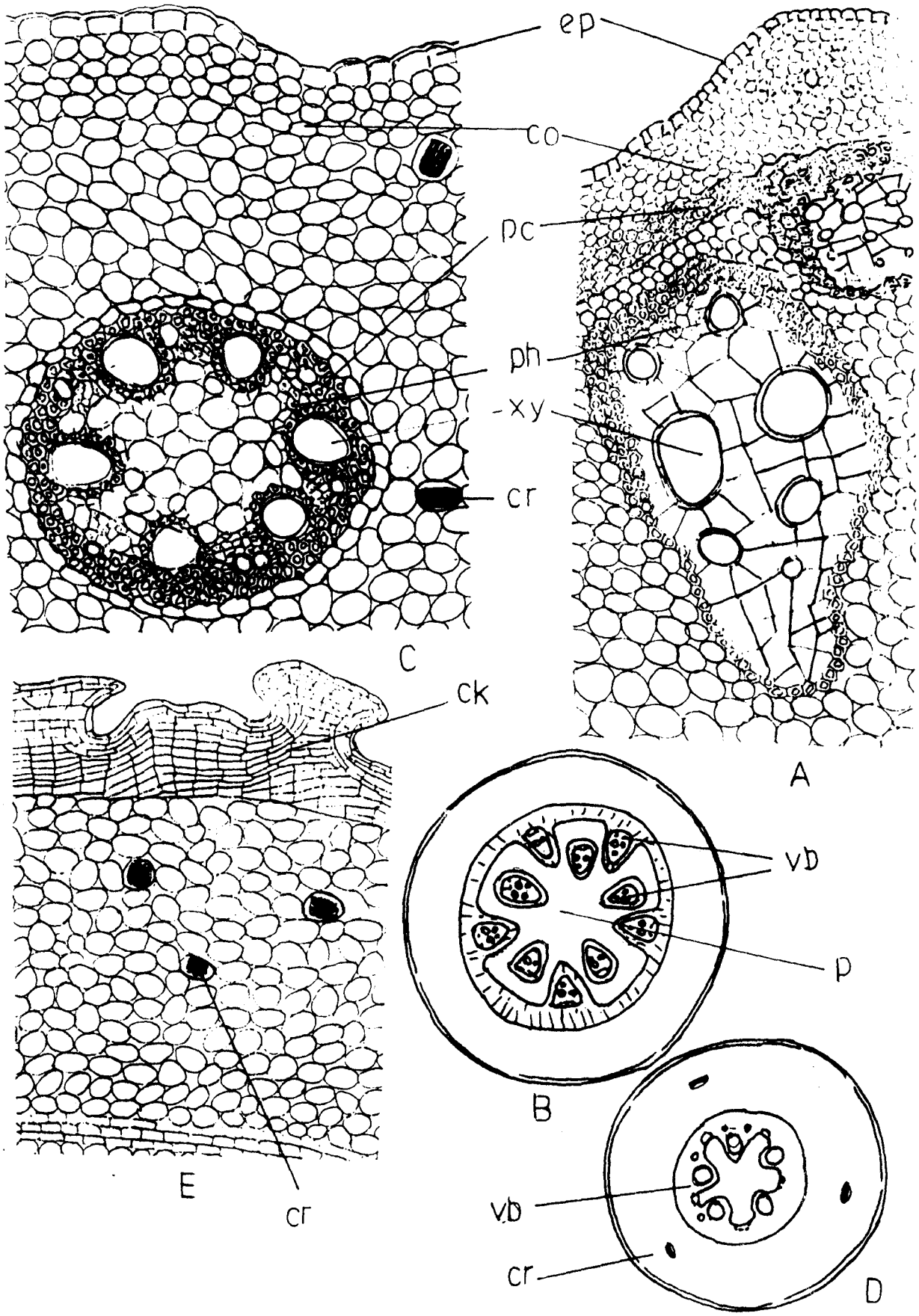

Fig: 34. ***D.prazeri.*** **A**.T. S of a part of stem **B** T. S of stem (Diagrammatic) **C**. T. S. of a part of root . **D.** T. S. of root (Diagrammetic) **E** T. S. of a part of yam.ck-cork, co-cortex, cr- crystals, ep-epidermal cells, p-pith pc-pericycle, ph-phloem, rh- root hair, vb-vascular bundle, xy-xylem.

SECTION B

Phytochemical investigation on the yams of different Dioscorca species with special emphasis on steroidal constituents

Materials and Methods

Materials yams of eight species of *Dioscorea* i.e. *D. alata*, *D.kamoonensis*, *D.arachidna*, *D.sikkimensis*, *D.bulbifera*, *D.esculenta*, *D.sativum* and *D.prazeri*.

Methods

Collection and preparation of yam

The yams of each species of *Dioscorea* were collected; and cleaned with water after clipping the roots. The excess of adhering water was removed. They were cut into small pieces using a chopping knife and sundried for several days until they became brittle. They were made to powder with an electric grinder.

Isolation and purification of phytosterol obtained from the yam of different species of Dioscorea. (Harborne; 1973)

Airdried and powdered yams of each of the species of *Dioscorea* was extracted separately in 3L soxhlet with petroleum ether (B.P. 60^0 - 80^0 C) for 48 hours. The extract was collected after filtration and was concentrated after heating to a small volume.

The concentrated solution was adsorbed on alumina in a glass column at room temperature and eluted with solvents starting from non-polar one to polar as follows:

Petroleum ether		
Petroleum ether	: Benzene	(3:1)
Petroleum ether	: Benzene	(1:1)
Petroleum ether	: Benzene	(1:3)
Benzene		
Benzene	: Chloroform	(3:1)
Benzene	: Chloroform	(1:1)
Benzene	: Chloroform	(1:3)

Chloroform
Chloroform : Methanol (3:1)
Chloroform : Methanol (1:1)
Chloroform : Methanol (1:3)
Methanol

Libermann - Burcharal test for sterols

The methanolic solutions (1 ml) of sample was treated with accetic anhydride (1 ml) and then with concenrated H_2SO_4.

Phosphoric acid for detection of steroid

1 volume 0-phosphoric acid was diluted with 1 volume of water

Antimony trichloride (Carr-price reagent) for steroids.

20% Antimony trichloride in chloroform.

Extraction isolation and purification of diosganin (Bammi and Randhawa 1975)

100 gm of yam of each species of *Dioscorea* was dried in an oven for 6 to 8 hours at 100°C for moisture estimation. About 20 g dried sample was blended thoroughly in a mixer with known amount of water (50 ml) for 5 minutes.

The slurry was hydrodysed with 11.3 (N) HCl. For 3 hours. The slurry obtained after hydrolysis was allowed to attain room temperature and filtered using vacuum in a buchnerfunnel. The residue was frequently wasthed with distilled water till the filtrate was free from acid as indicated by the use of litmus paper. The filtered residue was transferred to a petridish and dried in an oven at 10°C for 6 hours. It was extracted with petroleum ethed (b.p. 40 -60°C) in a soxhlet for 8 hours. The extracted solvent with diosgenin was concentrated to about 25 ml., chilled in ice and filtered. The diosgenin was obtained from mother liquor after filtration. The whole diosgenen was weighed after drying it in an oven for 2 hours at 100°C.

Characterisation of chemical compounds

The identity of different isolated chemical compound was confirmed by their chemical and physical data obtained from

(i) Melting point determination (m.p.)
(ii) Mixed melting point determination (m.m.p.)
(iii) Thin layer chromatography (TLC)
(iv) Paper chromatography (PC)
(v) Infrared spectrum (IR)

RESULTS

I. Isolation and characterisation of phytosterol in the yam of different species of *Dioscorea*

(i) *D. alata*

The petroleum ether (b.p. 60^0 - 80^0C) extract of the air dried powdered yams of *D.alata* was taken in a minimum volume of chloroform after evaporating the pet.ether to dryness and column chromatographed over alumina. The crystals obtained offer elution of the column with different solvents and their mixtures are represented in the following table 15.

Table 15 : The residue obtained from Petroleum ether extract of yam of d.alata during column chromatograhy.

Eluent	Subfraction number	Residue on evaporation
Pet. Ether.	1-15	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Oil
Do	16-20	No residue
Do	21-25	Do
Petther : Benzene (1:1)	26-30	Do
Do	31-35	Do
Do	36-40	Do
Ptether : Benzene (1:3)	41-45	Crystals
Do	46-50	Crystals
Benzene	51-55	Crystals
Do	56-60	No residue
Benzene : Chloroform (3:1)	61-65	Do
Do	66-70	Crystals
Bengene : Chloroform (1:1)	71-75	Crystals
Do	76-80	Crystals
Benzene : Chloroform (1:3)	81-85	No residue
Chloroform	86-90	Do
Do	91-95	Do
Chloroform : Methonol (3:1)	69-100	Do
Do	101-105	Do
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do
Do	131-135	Do

Subfraction : 41-55

The subfractions of the eluent were collected and eparated to a small volume when an appreciable amount of crystals (35 mg) having m.p. at 171-172^{0}C. It was recrystallised in chloroform methanol mixture and showed m.p. at 170^{0}C. It was soluble in benzene, chloroform, sparingly soluble in ethanol but remained insoluble in water. The plates (silica gel G) were developed which showed the Rf. values 0.28 in benzene : chloroform (40:60 v/v) and 0.60 in chloroform : acetone (99.1; v/v). These values coincided with those of authentic stigmasterol. These plates were sprayed with Liberman - Burchard reagent and heated in an. Oven having temperature at 85-90 ^{0}C for 15 minutes. The plates developed the characteristic blue colour at the Rf. region of the sterol of authentic stigmasterol. The IR λ max 3320, 1720, 1660, 1450, 1350, 1300, 1250, 1050, 950 cm^{-1} spectrum also confirmed the sterol as stigmasterol due to super imposible peaks. (Fig. 35).

Subfraction - 66-80

The collected subfractions were mixed and concentrated after evaporation under reduced pressure when crystals (100 mg) were obtained. The solid mass was dessolved in chloroform and recrystallised from chloroform methanol mixture. The melting point (m.p.) was determined to 149 ^{0}C. The isolated product was spolted on T.L.C. plate liti silica gel G as absorvent. The isolaed product was run in the solvent mixture of chloroform. Acetone (99.1) Rf.0.56. Benzene chloroform (40.00 v/v) Rs.0.44, Benzyl ethylacetate (90.1 v/v) Rf.0.60. the developed chromotogram was sprayed with 0-phosphoric acid followed by subsequent heating at 120 ^{0}C for 10 min. the spot on the plate showed characteristic linged colour. Coinciding with the authentic sample of cholesterol. The IR spectrum of the isolated product showed charcteristic peaks Xmas : 3400, 1700, 1675, 1380, 1050, 950, 850, 800 cm^{-1} similar to those of authentic sample of cholesterol. (Fig.36)

(ii) *D. kamoonenosis*

The petroleum ehter (b.p. 60 ^{0}C - 80 ^{0}C) extract of the air dried powdered yams of d.kumaonesis has taken in a minimum volume of chloroform after evaporating the petether to dryness and column chronatographed over alumina. The crystals obtained after elution of the column with different solvents and their mixture are represented in the following table.16.

Table 16 : The residue obtained from petroleum ether extract of yam of *D.kamoonensis* during column chromatography.

Eluent	**Subfraction number**	**Residue on evaportion**
Pet ether	1-5	Oil
Petether : Benzene (3:1)	6-10	Oil
Do	11-15	Oil
Do	16-20	Oil
Petether : Benzene (1:1)	21-25	No residue
Do	26-30	Do
Do	31-30	Do
Do	36-40	Do
Do	41-45	Do
Benzene	46-50	Do
Benzene : Chloroform (3:1)	51-55	Do
Do	56-60	Do
Do	61-65	Oil
Do	66-70	Oil
Benzene : Chloroform (1:1)	71-75	No residue
Do	76-80	Crystals
Do	81-85	Crystals
Do	86-90	Crystals
Benzene : Chloroform (1:3)	91-95	No residue
Chloroform	96-100	Crystals
Chloroform : Methanol (3:1)	101-105	Crtstals
Do	106-110	No residue
Do	111-115	Do
Chloroform : Methanol (1:1)	116-120	Do
Do	121-125	Do
Chloroform : Methanol (1:3)	126-130	Do
Methanol 1-5	131-135	Do

Subfraction : 76-90

The crystals were collected after evaporating the solvents and recrystallised repeadedly from acetone when small amount of crystal was deposited (60 mg). A small fraction of the isolated cystal has dissolved in hot methanol and subjected to TLC and has beem found to be identical in behaviour with the authentic. Sample of pure lanosterol showing Rs.0.79 (Chloroform acetone, 99:1, v'v) Rf.0.63 (Benzene : Chloroform, 40:60 v/v) and Rf. 0.71(Benzene : Ethyl acetate, 90:1, v/v) The isolated crystals showed positive test with Liebermann - Burchard reagent. Characterisation of the isolated chemicals was finally confirmed with m.p. (140° - 141 °C) mmp (undepressed). The IR spectrum of the compound has been examined. The absorption peaks and their assignments were identical with those of authentic samples of pure lanosterol (Fig.37).

Sub fraction : 96-105

The subfractions were mixed and concentrated after evoporation under reduced pressure to obtain crystals (30 mg). After recrystallisation from chloroform - methanol it was identified as choresterol after comparing m.p. Chromotographic behaviour and IR spectrum of the isolated product with those of authentic sample of cholesterol that have been represented earlier (Fig.36)

(iii) *D. arachidna*

The petroleum ether (b.p. 60 oC - 80 oC) extract of the airdried powdered. Yams of D.arachidna was taken in a minimum volume of chloroform after evaporating the pet ether to dryness and column chromatographed over alumina. The crystals obtained offer elution of the column with different solvents and this mixtures are represented in the following table 17.

Table 17 : The residue obtained from petroleum ether extract of yam of *D.arachidna* during column chromatography.

Eluent	Subfraction number	Residue on evaporation
Pet. Ether.	1-5	Oil
Do	6-10	Oil
Pether : Benzene (3:1)	11-15	Oil
Do	16-20	No residue
Do	21-25	Do
Do	26-30	Do
Petther : Benzene (1:1)	31-35	Do
Do	36-40	Do
Do	41-45	Do
Ptether : Benzene (1:3)	46-50	Do
Do	51-55	Oil
Do	56-60	Oil
Benzene	61-65	Oil
Do	66-70	No residue
Benzene : Chloroform (3:1)	71-75	Do
Do	76-80	Do
Bengene : Chloroform (1:1)	81-85	Do
Do	86-90	Do
Benzene : Chloroform (1:3)	91-95	Do
Chloroform	96-100	Crystal
Do	101-105	Crystal
Chloroform : Methonol (3:1)	106-110	Crystal
Do	111-115	Crystal
Chloroform : Methanol (1:1)	116-120	Crystal
Do	121-125	Crystal
Chloroform : Methanol (1:3)	126-130	No residue
Do	131-135	Do
Methanol	136-140	Do

Sub fractions : 96-125

The collected subfractions were mixed and concentrated after evaporation under reduced pressure when crystals (90 mg) were obtained. The isolated products were recrystallised from chloroform methanol mixture. The m.p. determination, TLC, IR spectrum of the isolated product were determined and in all respects the isolated product show similar behaviour with those of authentic cholesterol (Fig. 36) and the observations of which have been represented earlier in this part of work.

(iv). *D.sikkinuris*

The petroleum ether (b.p. 60o - 80oC) extract of the air dried powdered yams of *D. sikkimensis* was taken in a minimum volume of chloroform after evaporating the pet ether to dryness and column chromatographed over alumina. The crystals obtained after elution of the column with different solvents and their mixture are represented in the following table 18.

Table 18: The residue obtained from petroleum ether extract of yam of *D.sikkimensis* during column chromatography.

Eluent	**Subfraction number**	**Residue on evaporation**
Pet. Ether.	1-5	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Crystals
Do	16-20	Crystals
Do	21-25	Crystals
Petther : Benzene (1:1)	26-30	Crystals
Do	31-35	Crystals
Do	36-40	Crystals
Ptether : Benzene (1:3)	41-45	No residue
Do	46-50	Do
Benzene	51-55	Do

Contd.....

Eluent	Subfraction number	Residue on evaporation
Do	56-60	Crystals
Benzene : Chloroform (3:1)	61-65	Crystals
Do	66-70	Crystals
Bengene : Chloroform (1:1)	71-75	Crystals
Do	76-80	No residue
Benzene : Chloroform (1:3)	81-85	Do
Chloroform	86-90	Do
Do	91-95	Do
Chloroform : Methonol (3:1)	96-100	Do
Do	101-105	Do
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do

Subfraction - 11-40

The subfractions of the eluent were collected and evaporated to small volume when an appreciable amount of crystals (85 mg) were deposited. The ; isolated product was observed to be positive to liebermann - Burcharded test and was identified as stigmasterol, following the procedure mentioned earlier in this part of work (Fig.35).

Subfraction 56 - 75

The subfraction of the eluent were collected and evaporated to small volume when an amount of 90 mg of cyrstals. Were obtaianed and which was observed to be positive to Libermann - Burchard test. Following the procedure as representation earlier, the isolated product was identified as cholesterol (Fig.36).

(v). *D. bulbifera*

The petroleum ether (b.p. 60^0 - 80^0C) extract of the air dried powdered yams of *D. bulbifera* was taken in a minimum volume of chloroform after

evaporating the pet ether to dryness and column chromatographed over alumina. The crystal obtained after elution of the column with different solvents and their mixture are represented in the following table19.

Table 19: The residue obtained from petroleum ether extract of yam of *D.bulbifera* during column chromatography.

Eluent	**Subfraction number**	**Residue on evaporation**
Pet. Ether.	1-5	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Oil
Do	16-20	Crystals
Do	21-25	Crystals
Petther : Benzene (1:1)	26-30	Crystals
Do	31-35	No residue
Do	36-40	Do
Ptether : Benzene (1:3)	41-45	Do
Do	46-50	Do
Benzene	51-55	Do
Do	56-60	Do
Benzene : Chloroform (3:1)	61-65	Do
Do	66-70	Do
Bengene : Chloroform (1:1)	71-75	Do
Do	76-80	Do
Benzene : Chloroform (1:3)	81-85	Crystals
Chloroform	86-90	Crystals
Do	91-95	Crystals
Chloroform : Methonol (3:1)	96-100	Crystals
Do	101-105	No residue
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do
Do	131-135	Do

Sub fraction : 16-30

The subfraction of eluent were collected and evaporated to small volume. When an appreciable amount (75 mg) of crystals were obtained.

The isolated product was studied from chemical point of view. It was identified to be stigmateral following the procedure mentioned earlier. (Fig.35)

Subfraction 81-100

The subfractions of eluent were mixed up and evaporated to small volume when an amount of 50 mg of crystals were obtained. It was observed to be positive to lieberman Burchard test. The isolated product was identified to be cholesterol following the procedure involving observation on m.p. TLC and IR spectrum as mentioned earlier. (Fig. 36).

(vi). *D.esculenta*

The petroleum ehter (b.;. 60o - 80o) extract of the air dried powdered yams of D.esculenta was taken in a minimum volume of chloroform after evaporating the pet ether to dryness and column chromatographed over alumina. The crystals obtained after elution of the column with different solvents and their mixture are represented in the following table 20.

Table 20 : The residue obtained from petroleum ether extract ofl yam of d.esculenta during column chromatography.

Eluent	Subfraction number	Residue on evaporation
Pet. Ether.	1-5	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Crystal
Do	16-20	Crystals
Do	21-25	Crystals
Petther : Benzene (1:1)	26-30	Crystals
Do	31-35	No residue
Do	36-40	Do

Contd...

Eluent	Subfraction number	Residue on evaporation
Ptether : Benzene (1:3)	41-45	Do
Do	46-50	Do
Benzene	51-55	Crystals
Do	56-60	Crystals
Benzene : Chloroform (3:1)	61-65	Crystals
Do	66-70	Crystals
Bengene : Chloroform (1:1)	71-75	Crystals
Do	76-80	Crystals
Benzene : Chloroform (1:3)	81-85	No residue
Chloroform	86-90	Do
Do	91-95	Do
Chloroform : Methonol (3:1)	96-100	Do
Do	101-105	Do
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do

Subfraction 11-30

The subfractions of the eluent were collected and evaporated to a small volume when an appreciable amount of crystals (45 mg) was deposited. Pure crystals were obtained from chloroform methnal mixture having m.p. 138 - 140^{0}C. This was recrystallised from acitone and colourless needles of crystals were obtained. A small fraction of the crystals was dissolved in hot methanol and subjected to TLC on silicagel G and was found to be identical in behaviour with the authentic sample of pure sitosterol (Chloroform : acetone, 99:1, v/v, Rf. 0.54; Benzene : Chloroform, 40:60, v/v, Rf.0.46; Benzene : Eehylacetate, 90:10, v/v, Rf.0.59). The developed chromatogram was sprayed with O-phosphoric acid and the plate was heated at 120^{0}C for 10 mins. The; bluish spots of authentic sample of sitosterol as well as isolated crystals produced greyish brown floures cence on the developed chromatogram under UV light.

The IR spectrum of the isolated crystals showed characteristic peaks at λ max 3310, 1740, 1710, 1460, 1380, 960 cm^{-1}. Its characteristic peaks and their assignments were identical with those of authentic sitosterol (Fig. 38).

Subfraction 51-80

The subfractions of eluent were collect and evaporated, to small volume when an amount of 55 mg. of crystals were obtained. It was observed to be positive to Liebermann - Burchard test. The isolated - phytosterol was identified to be chlesterol on the basis of the procedure involving physical and chemical parameters already represented elsewhere in this part of work. (Fig.36)

(vi). *D.sativum*

The petroleum ether (b.p. 60-80oC) extract of theair dried powdered yams of D.sativum was taken in a minimum volume ofchloroform offer evaporating the pet ether todryness and column chromatographed over alumina. The cyrstals obtained after elution of the column with different solvants and their mixture are represented in the following table 21.

Table 21 : The reside obtained from petroleum ether extract of yam of D.sativum during column chromatography.

Eluent	Subfraction number	Residue on evaporation
Pet. Ether.	1-5	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Oil
Do	16-20	No residue
Do	21-25	Do
Petther : Benzene (1:1)	26-30	Do
Do	31-35	Do
Do	36-40	Do
Ptether : Benzene (1:3)	41-45	Do
Do	46-50	Do

Contd...

Eluent	Subfraction number	Residue on evaporation
Benzene	51-55	Do
Do	56-60	Do
Benzene : Chloroform (3:1)	61-65	Crystals
Do	66-70	Crystals
Bengene : Chloroform (1:1)	71-75	Crystals
Do	76-80	No residue
Benzene : Chloroform (1:3)	81-85	Crystals
Chloroform	86-90	Crystals
Do	91-95	No residue
Chloroform : Methonol (3:1)	96-100	Do
Do	101-105	Do
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do

Subfractions : 61-75

The subfractions were mixed and evaporated todryness. The residue was disolved in chloroform and methanol was added. Crystals appeared after sometime. They were separated and recrystalised to obtain the natural product of 70 mg. It was identified to be stigmasterol after following the procedure represented earlier. (Fig. 35).

Subfraction : 81-90

The subfractions were collected and evaporated to small volume when an amount of 30 mg. of crystals were obtained and which has identified as cholesterol after comparing the m.p. chromatographic behaviour at IR spectrum of the isolated product with those of authentic sample. (Fig.36).

(vii). *D. prozeri*

The petroleum ether (b.p. 60 - 80^{0}C) extract of air dried powdered yams of *D.prazeri* was taken in a minimum volume of chloroform after evaporating

the pet ether to dryness and column chromatographed over alumina. The crystals obtained after elution of the column with different solvents and their mixture are represented in the following table 22.

Table 22 : The reside obtained from petroleum ether extract of yam of *D.prozeri* during column chromatography.

Eluent	Subfraction number	Residue on evaporation
Pet. Ether.	1-5	Oil
Pether : Benzene (3:1)	6-10	Oil
Do	11-15	Oil
Do	16-20	Crystals
Do	21-25	Crystals
Petther : Benzene (1:1)	26-30	Crystals
Do	31-35	No residue
Do	36-40	Do
Ptether : Benzene (1:3)	41-45	Do
Do	46-50	Do
Benzene	51-55	Crystals
Do	56-60	Crystals
Benzene : Chloroform (3:1)	61-65	No residue
Do	66-70	Do
Bengene : Chloroform (1:1)	71-75	Do
Do	76-80	Do
Benzene : Chloroform (1:3)	81-85	Do
Chloroform	86-90	Oil
Do	91-95	Oil
Chloroform : Methonol (3:1)	96-100	No residue
Do	101-105	Do
Chloroform : Methanol (1:1)	106-110	Oil
Do	111-115	Oil
Chloroform : Methanol (1:3)	116-120	No residue
Do	121-125	Do
Methanol	126-130	Do

Subfractions 16-30

The subfractions were mixed and the total volume was reduced to small volume and kept over night. The soid was dissolved in chloroform and mixed with methanol and kept for one day. Thecrystals were separated out. The amount was observed to be 40 mg. It was identified as sitosterol following the procedure involving the ditermination of mp. TLC and IR spectrum mentioned earlier. (Fig. 38).

Subfractions : 51-60

The sub fractions were mixed and the solvent was evaporated to obtain residue. It was dissolved in chloroform and methanol was added. It was kept over night to obtain crystals. (30 mg). It was identified as cholesterol after following the methodology represented elsewhere in this part of work.

II. Isolation and characterisation of natural product obtained from petroleum ether extract of acid hydrolysed yam of different species of Dioscorea.

Following the method proposed by Bammi and Randhawa (1975), crude residue was obtained after evaporating petroleum ether which, was used for extraction of acid hydrolysed yam of each species of *Dioscorea* a separately. The solid mass obtained from yam of each of the species was dissolved in chloroform and methanal was added. It was kept overnight is cold and white waxy plated crystals were obtained. The crystals showed m.p. 206-208^{0}C. It was subjected to TLC on silica gel G in solvents such as Chloroform. Acetone (4:1 v/v) Rf.0.58; Hexane : Acetone (4:1, v/v) Rf.0.32. The plate showed purple spots after spraying Antimony trichloride in cone HCL and subsequent heating at 110^{0}C for 10 minutes. The observation was very much similar to those with authentic diosgenin. Identification of diosgenin was further confirmed by the superimpossible IR Spectrum (in Nujol) at λ max 3440, 3400, 1375, 1250, 1050, 900 cm^{-1} for both the isolated product and authentic diosgenin (Fig.39)

Table 23: Production of diosgenin in the yam of different species of Dioscorea.

Species	% ofdiosgenin on dry wt. basis
D. alata	0.07
D. kamoonensis	0.50
D. arachindna	0.70
D. sikkimensis	0.60
D. bulbifera	0.90
D. exculenta	0.06
D. sativum	1.50
D. prazeri	2.50

Discussion

India's steroid industries are at present based mainly on two species of *Dioscorea* i.e. *D.prazeri. Prain* and *Burkill* and *D.deltoidea* Wall. In addition, a very few species of *Dioscorea* have been introduced in the country as the source of raw materials for the production of diosgenin used as a principal precursor compound for the synthesis of corticosteroid drugs and sex hormones as active constituents of oral contraceptives. Collection and supply of these materials from their natural habitats for the last few decades have reached the natural population to a very scanty level. (~~Bammi et al 1969~~; Chakraborty and Roycoudhury 1974). Reackless and ruthless collections leading little scope for their regeneration in natural condition have brought these plants even upto the rank of endangered spceis. Considering the large demands of these material, searching of alternative sources of diosgenin is being felt now a days.

All together eight different species of *Dioscorea* growing wildly in Darjeeling and Sikkim Himalayas have been collected. These are *D.alata. D.kamoonensis* kunth, *D.arachidna* Prain and Burkill. *D.sikkimensis Prain* and *Burkill, D.bulbifera* L. *D.esculenta* (Roxb) Prain and Burkill, *D.sativum* L and *D. prazeri Prain* and *Burkill.* It has been observed that all these species are being used traditionally by the locally available tribal people in the region of Darjeeling and Sikkim Himalayas. But the plant parts of these dioscoreas are

very much similar in their external appearance and it is very difficult to identify them properly. Uinder these circumstances it is feltnecessary to identify them from the view point of pharmacognosy which includes the study of botanical and chemical aspects of plant.

Information about the pharmacognostic study of *Dioscorea* is meagre. X.freda and Cecilin (1990) carried out histochemical analysis of yam of different species of *Dioscorea* but they gave much importance only to study the "Crystalliferous sheaths" showing variation in oxalate crystals and starch is different species of *Dioscorea.* Bhatt et al (1990) on the other hand studied specialisation of vessels in the yam of *Dioscorea* sp. Philip et al (1980) studied seven species of *Dioscorea* from pharmacognosy point of view. They studied the tubers of different species of *Dioscorea* considering the identifying character based on the microscopic study of powdered tubers with special interest on crystals, starch grains, xylem vessels, fibre etc.

Thus in this part of work much importance has been given not only to the yam but also to other plart pacts like leaf, stem and root. Organoleptic study including macroscopic appearance of different species of *Dioscorea*, their shape, colour, feeling by touch, taste, marking and colour has been carried out. Table 8 and 9 show that there are marked variation in organoleptic features amongst eight species of *Dioscorea.* Some macroscopic features of different species have been worked out and morphological appearance of *D.alata* (Fig.11,12) *D. kamoonensis* (Fig.14,15) *D.arachidna* (Fig.17,18) *D.sikimensis* (Fig.20,21) *D.bulbifera* (Fig.23,24) *D.esculenta* (Fig.26,27) *D.sativum* (Fig.29,30) and *D. prazeri* (Fig.32,33) have been represented. Macroscopic evaluation of different plant parts of *Dioscorea* sp. has been worked out following the schedule of Johansen (1940). Some important microscopic features of leaf (Table 10) stem (Table 11), root (Table 12), and yam (Table 13) of all the eight species of *Diascorea* have been represented. In connection with quantitative microscopy two criteria such as stomatal index and pallisade ratio in the leaf of all the species have been worked out. From the table 14 it appears that *D. prazeri* and *D. sikkimensis* show the minimu (19) and maximum (30) values for stomatal index respectively. On the other hand *D.esculenta* and *D.sativum* show the

minimum (30) and maximum (59) value for pallisade ratio respectively. A key has been evolved based on observations on morphological and histological differences in leaf, stem, root and yam (Chart 1). The key is expected to help in providing an easy and quick identification of these species for their purposeful utilisation in our society.

Pharmacognosy is closely related to both botany and plant chemistry and indeed both originated from the earlier scientific studies on medicinal plants. For a long time the subject was mainly based on botanical aspects of the plant mainly concerned with the description and identification of drugs. Such branches of pharmacognosy are still of fundamental importance but rapid development in other area, particularly phytochemistry have enormously expanded the subject. The concept that plants can be identified and classified on the basis of their chemical constituents is not new that it is only during the last few decades that modern techniques of isolation and characterisation have led to the chemical screening of large number of plant samples. Compared with morphological charectars chemical constituents in plants are often more fundamental significance for identification of plants (Evans 1997).

For a long time different species of *Dioscorea* have been noted to yield steroidal saponins. Which are having with high molecular weight and high polarity. These glycosides after being subjected to acid hydrolysis yield aglycone (Sapogenin) Biosynthetically they are related to other steroidal constituents and specially the phytosterols. It is expected that different species of *Dioscorea* may contain saponin and phytosterol as complex mixture. For this reason isolation and characterisation of phytosterol in different species of *Dioscorea,* under consideration, along with diosgenin have been undertaken.

Phytosterols and diasgenin have been isolated from the yams of different species of *Dioscorea* following the methods of Harborne (1973) and Bammi and Randhawa (1975) respectively. Cholesterol (Fig. 36) have been observed to be present in the yams of all the species of Diascorea under consideration (Table 15 to 22). Stigmasterol (Fig.35) is common in the yam of all the four species such as *D.alata* (Table 15), *D. sikkimensis* (Table 18), *D.bulbifera* (Table 19) *D. sativum* (Table 21). But lanosterol (Fig.37) has been isolated

CHART-1

Key for quick identification of different species of *Dioscorea* from pharma cognosy point view

A. Leaf Simple

B. Phyllotaxy alternate

C. Vascular bundles are embeded inthe sclerenchymatons pericycle in root

D. Shape of leaf, cordate

E Stem is polygon in cross section but pentagon in the basal part of the petiole. Stigma sterol is persent in the yam, round in shape ..... *D. sativum*

E_1 Stem and basal part of petiole are circular in cross section, sitosterol is present in the yam ..... *D. esculenta*

D_1 Shape of leaf, ovate lanceolate, basal part of petiole cordate in cross section with a sharp knotch, sitoterol is present in the yam branched, cylindrical is shape ..... *D. prazeri.*

B_1 Phyllotaxy opposite superposed

C_1 In stem vascular bundles are aranged in two rings, some are embeded in a ring of pericycle, the others are distributed towards the pith.

D_2 Stem is triangular in cross section with curved arms but dome shaped in the basal part of petiole, there is projection of tissue below the vascular bundle in the midrib of leaf, stigmasterol is present in the yam, oblong in shape ..... *D. sikkimensis*

D_3 Stem is pentagon in cross section with slightly curved arms. In root, vascular bundle touches the pericycle with protoxylam, metaxylem part is free. Basal part of petiols is pentagon in cross section; stigmasterol is present in the yam, cylindrical..... *D. bulbifera.*

C_2 In stem,vascular bundles are embeded in a ring of pericycle, stem is elliptic in cross section but dom shaped it the basal part of petiole. Stigmasterol is present in the yam, cylindrical, much broader at the proximal end. ..... *D. alata*

A_1 Leaf, compound.

B_2 In stem vascular bundles are arranged in two rings, some are embeded in the pericycle and others are distributed more towards the pith

C_3 In root vascular bundles are embeded in the pericycle, but metaxylem part is freely projected to wards the pith. Basal part of petiole is semicircular in cross section and the midrib region is very much projected from the laminar surface, Lanosterol is present in the yam, cylindrical with round proximal and distal ends ..... *D. kamooneusis.*

C_4 In root protoxylem and metaxylem of vascular bundles are totally embeded in the pericycle. Basal part of petiole is circular with an are of straight line the midrib region is not projected cholesterol is present in the yam, round. .... *D. arachidna.*

from *D. kamoonensis* (Table 16) and sitosterol (Fig.38) from *D.esculenta* (Table 20) and *D.prazeri* (Table 22). It is very interesting to note that besides cholesterol (Fig.36) no other sterol has been detected in *D.arachidna* (Table 17). From previous record it appears that Lin and yam yong (1985) reported the presence of sitosterol in the yam of *D.septamoilea*. Savikuri Fodulovic et al (1998) isolated phytosterol in callus line of wild. *D.balcamea.*

The generally accepted path way for steroid biosysthesis in plants was outlined by Kaneko et al (1976). According to the biosynthetic path way acetyl CoA was treated as the starting point which was converted to cholestorol via mevalonic acid and squalane.

Cholesterol was considered for many years as the typical animal sterol and was differentiated on that basis from the plant or phytosterol. On the other hand cycloartenol is considered typical for plant as this compound has not be found in animals (Jacokson 1970). In 1958 cholesterol was discovered in lower plants (Tsuda et al, 1958) and subsequently its presence was shown in micro organisms and higher plants (Heftmann, 1967).

In biosynthetic experiments cholesterol contained one quarter to one third of all the radioactivity incorporated into sterol with mevalonic acid 2-14 C as substrate. By weight cholesterol accounted for only three percent of the sterol mixtue. Consequently, it appeared that cholesterol was rapidly formed and metabolised (Jacobson, 1970). The ability of cholesterol to serve as precursor for other 27 carbon sterol was shown by its conversion to diosgenin. (Bennett and Heg & Mann, 1965; Tschesche and Hulpke 1966). The question arose as to which compound constitutes the first stable sterol after cyclozation of squalane. Wool ward and Block (1958) proposed a mechanism in which Lanosterol was considered to be the first intermediate in connection with the process.

According to biogenetic scheme presented by Schreiber (1968) and Schutte (1969) and intermediate giving rise to diosgenin was postulated as 16 - hydroxycholesterol derivative having unsaturations at the side chain positions 22 and 25 oxidation of the double bonds by plant metabolism could then lead to 16 - dihydrokryptogenin which in turn when cyclized would afford diosgenin.

According to Stohs et al (1974) sitosterol also served as precursor in the formation of saponin. With sitosterol however, removal of a two carbon unit for C-24 must preceed oxygenation. The sequence in which oxygen is introduced at position 16,22 and 26 is unanswered (Grunwald, 1980) but indirect evidence strongly suggested that oxygenetion at C-26 was first step towards the formation of saponin (Bennett et al 1970). According to Johnson et al (1964) formation of stigmasterol is assumed to occur through sitosterol by enzyme 22,23-dehydrogenase (Johnson et al. 1964); Rowe 1965), however much of the evidence is indirect. Radioactive mevalonic acid is first detected in sitosterol, and with longer incubation the specific activity of sitosterol decreases while that of stigmasterol increases (Knapp and Nicholas 1971; Bush and Grunwald 1973) . Direct conversion of sitosterol to stigmasterol however is low, and all attempts to demonstrate that this is the major path way for stigmasterol biosynthesis are unconvincing (Waters and Johnson, 1965; Bennett and Heftman 1969).

As diosgenin and various phytosterols are biosynthelically related, it is expected therefore that different sterols may be available in different species of *Dioscorea* according to their genetic make up.

As regards other steroidal constituent all the species of *Dioseroea* have been observed to contain diasgenin though in variable quantity. (Table 23). Negligible amount of diosgenin i.e. o.07% and 0.06 on dry wt. basis have been isolated from the yam of *D.alata* and *D. esculenta* respectively. The table 23 also show that very low amount of diosgenin is present in the yam of *D.kamoonensis* (0.50%) *D. arachidra* (0.70%) *D. skkimensis* (0.60%) and *D.bulbisfera* (0.75%). Besides an appreciable amount of diosgenin is observed to be present in *D. sativum* (1.35%) and *D. prazeri* (2.30%). Kunithapadam (1982) reported the presence of 1 to 3% of diosgenin in the yam of *D. prazeri* available in North Eastern Region in India and Pal and Chakravarty (1976) isolated 1.7-2.0% of diosgenin in the yam of the same plant but they did not mention the place of collection of the plant material. But the presence of 1.35% of diosgenin on dry wt basis in the yam of *D.sativum* (Table 23) may be treated as the first time to report and such an amount may be treated as and alternative source of diosgenin in the region.

Savikin - Fodulovic et al (1998) worked out the biosynthetic relationship amongst diasgenin and phytosterol with the help of tussue culture technique. The obtained callus lines from awild *D. balcanica* cultivar, differed in diasgenin as well as in phytosterol content the observed that light/dark condition affected the amount of diosgenin in callus line while quantitative differences in phytosterol amount were less pronounced. There were quantitative differences in their amount between callus grown on light and in the dark. The addition of cholesterol in culture medium increased the amount of both diosgenin and phytosterol sugesting the role of cholesterol in the biosynthetic process of diosgenin and phytosterol.

There may be quantitative difference of all the natural products due to environmental factors but the qualitative character like availability of various phytosterol in the yam of different species of *Dioscorea* is genetically fixed and may serve as important charcter for identification of different species of *Dioscorea in* the line of their investigation from pharmacognosy point of view.

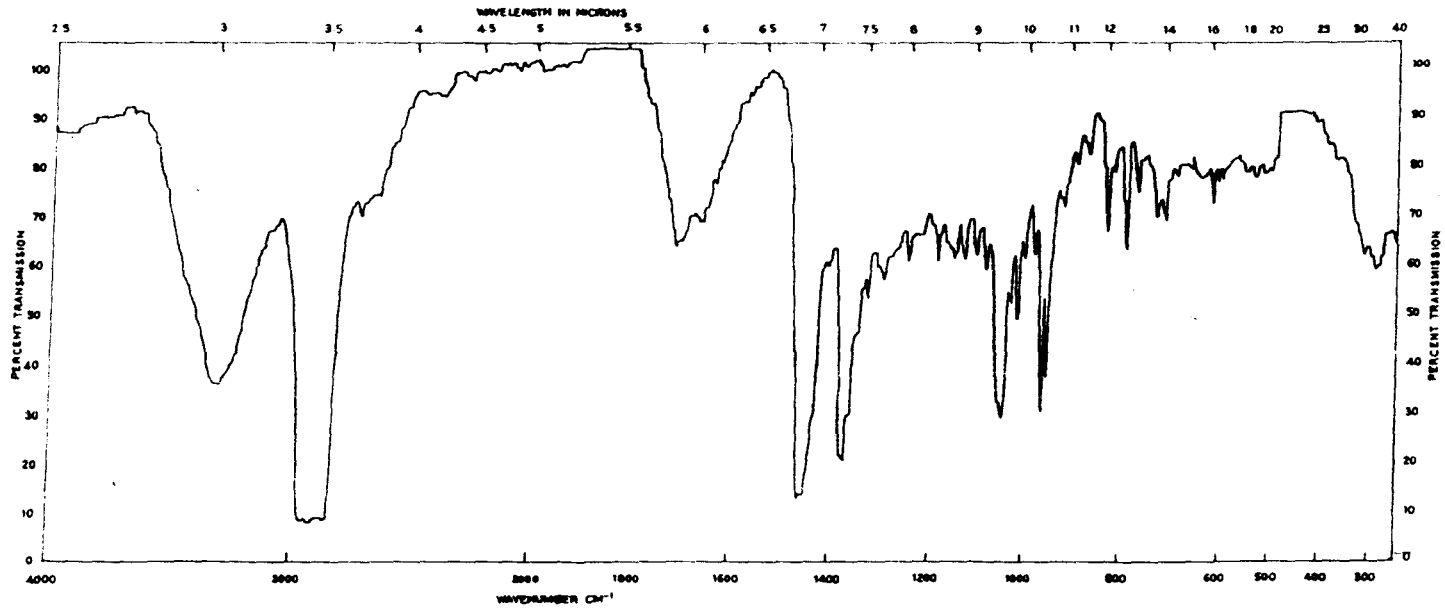

Fig. 35. IR - spectrum of stigmasterol.

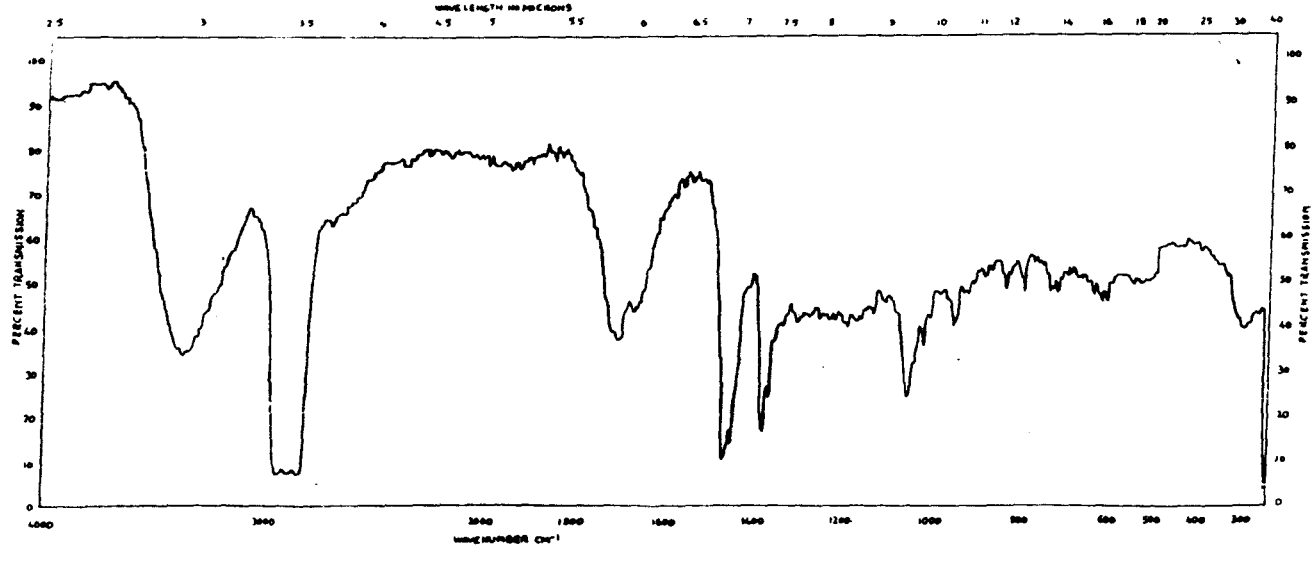

Fig. 36 IR spectrum of cholesterol

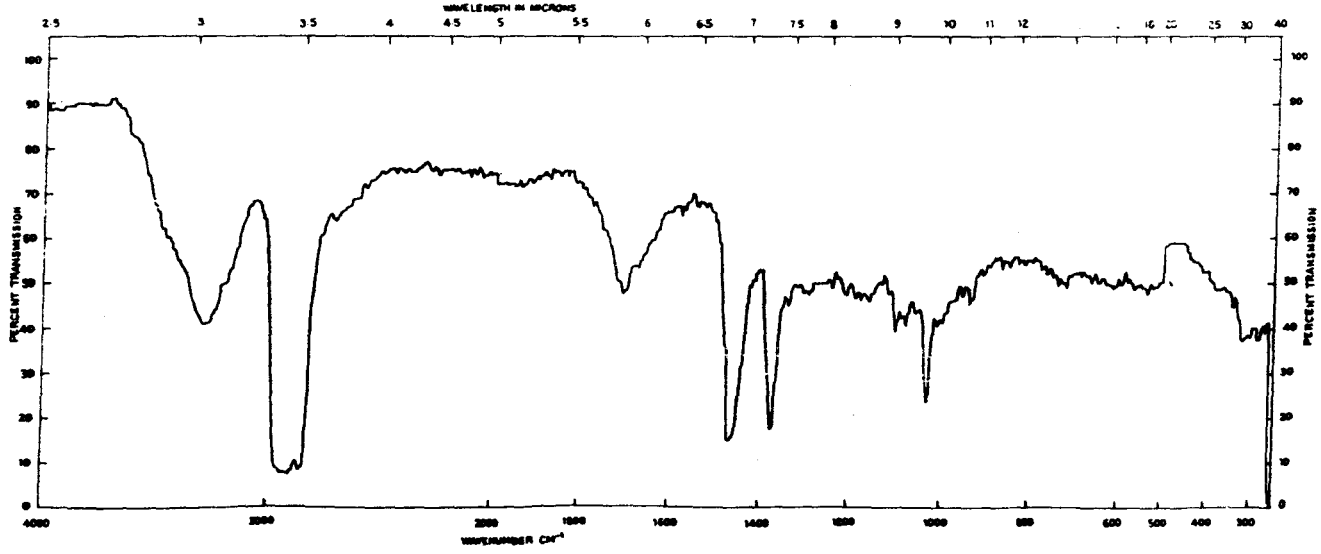

Fig. 37 1R spectrum of lanosterol.

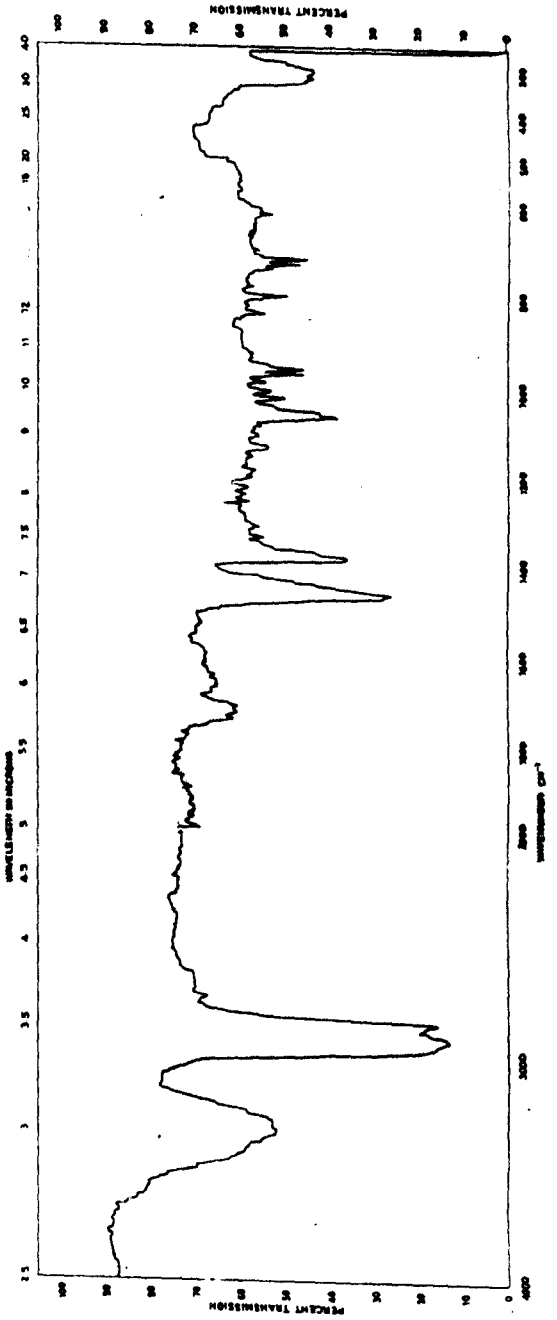

Fig. 38 IR spectrum of sitosterol.

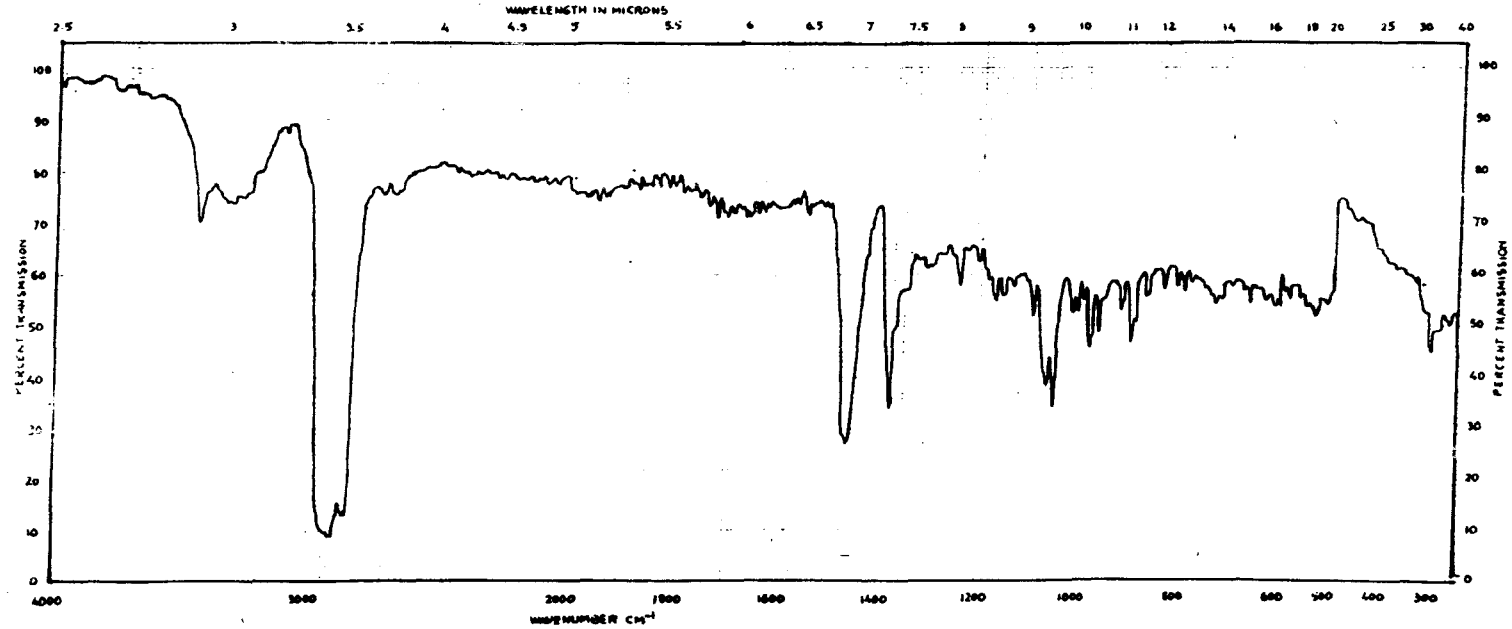

Fig. 39 IR spectrum of diosgenin.

SUMMARY

Eight different spices of *Dioscorea,* such as *D. alata* L, *D. kamoonensis* Kunth, *D. arachidna* Prain & Burkill, *D. sikkimensis* Prain & Burkill, *D. sativum* L and *D. prazeri* Prain & Burkill have been studied from. Pharmacognosy point of view to include botanical and chemical charactors.

Different parts of the plant such as, leaf, stem, root and yam have been studied for their identification on the basis of organoleptic, macroscopic and microscopic characters.

In connection with orgenoleptic study the yams of *D.alata,D. kamoonensis D. sikkimensis, D. bulbiferea* have been observed to produce no charactristric smell though *D. arachidna, D. sativum, D. esculenta* and *D. prazeri* have charactristric smell. All these species differ in shape, colour, markings of different parts. The yam of these species have also been observed to differ in feeling to touch specially in connection with the yam.

As regards macrosopic characters, *D. sativum D. esculenta* and *D. prazeri* have simple leaf with alternate phyllotaxy though opposite supurposed has been observed in *D.alata, D. bullifera* and *D. sikkimenis*.Compound leaf has been observed in *D. kamoonensis* and *D. arachidna.* All these species have been noted to differ in shape of leaf and yam.

Microscopic observations on leaf, stem, root and yam of all the species have been made. They have been noted to differ in the size of epidermal, pallisade and spongy cells type of stomata, shape at the basal region of petiole, out line of stem, epidernal hairs, thickness of pericycle in stem and root, arrangements vascular bundles, distribution of idioblasts containg ascicular crystals. Characterristic features in connection with the projection of tissue below the vascular bundle in midrib region of leaf have also been noted.

All the species have been observed to differ in some characters such as stomatal index and pallisade ratio of leaf.

In chemical aspects much emphasis has been given on isolation and charactarisation of phytosterols in the yam of eight species of *Dioscorea.*

Following conventional method of phytochemical analysis, dried powder of yam of all the species have been subjected to soxhlet extractions. with petroleum ether for isolation and identification os sterols.

They were identified, after comparing the m.p. - PC, T.L.C.and I.R.spectrum of the isolated product with those of anthentic samples of sterols. Stigmasterol, has been observed to be present in *D.* alata, *D. sikkimensis, D. bulbifera,* and *D.sativum*, lanosterol in *D. kamoonensis* and sitosterol in *D. esculenta.* and *D. prazeri* Cholesterol is present in all the species including *D. arachidna.*

Estimation of diosgenin has been made following the conventional method of pteroleum other extration of acid hydrolysed yam. *D.alata* and *D. esculenta* have been observed to contain 0.07 and 0.06 present on dry weight basis respectively. The percentage yield of diosgenin in other species are *D. kamoonensis* (0.50%) *D. sikhimensis* (0.60%) *D. arachidna* (0.70%) *D. bullifera* (0.75%), *D. sativum* (1.35%) and *D. prazeri* (2.30%).

On the basis of botanical and chemical characters, a key has been prepared, for quick identifications of the species, available in Darjeeling and Sikkim Himalayas.

CHAPTER IV

A NEW AND RAPID COLORIMETRIC METHOD FOR QUANTITATIVE ESTIMATION OF DIOSGENIN IN THE YAM OF DIFFERENT SPECIES OF *Dioscorea.* AVAILABLE IN THE REGION

Introduction

Out of various steroidal precursus diosgenin has been noted to be the most important source of raw material for the synthesis of steroid drugs. (Applezwig, 1962; Bhatnagar and Puri, 1974). The discovery that diosgenin found in the yam of *Dioscorea* can be converted to progesterone (Marker et al 1947) has lead to the production of oral contraceptives on commercial scals. Since then different species of *Dioscorea* yielding diosgenin has so far been utilised for a considerable period of time.

The situation started changing after that period when rising prices and uncertainty of the availability of *Dioscorea* yams caused shortage of steroid drugs based on diosgenin (Aplezweig, 1`969; Love, 1976). For this reasons very recently much importance has been given on searching of alternative source of diosgenin and for which an easy and rapid method for quantitative estimation of diosgenin is required.

Methodology sofar used for the estimation of diosgenin are gravimetric (Selvaraj, 1971) gas liquid chromatography (Tang et al 1978, Glyzine et al 1981) densito metric method using TLC scanner (Gunawan et al 1994). Paseshnichenko et al (1978) utilised colorimetric method for the determination of glycoside of diosgenin. There is no information available in connection with the utilisation of a rapid colorimetric method for quantitative estimation of diosgenin from the yam of *Dioscorea* on microscale. Here an attempt has been made to work out a rapid colorimetric method for quantitative estimation of diosgenin taking minimum amount of plant tissue.

Materials and Methods

Material

Yams of *D.alata, D. kamoonensis, D.arachidna, D.sikkimensis, D. bulbifera, D.esculenta, D.sativum and D.prazeri.*

Method

Collection and preparation of yam of *Dioscorea*

Yams of different species of *Dioscorea* were collected and washed with water to free it from soil particles. They were cut into pieces, sundried and made to powder with the help of a grinder machine. The dry powdered samples were used for extractron of diosgenin.

Extraction of diosgenin from powdered yam of Dioscorea

The powdered sample of yam was refluxed with chloroform for 15-20 minutes and filtered. The filtrate contained free diosgenin. The chloroform part was concentrated. The residue of plant tissue obtained after filtration was dried to free it from chloroform and was subjected to acid hydrolysis with 11.3 (N) HCl for 5 hours and neutralised. The solid matter was dried and extracted with petroleum. ether. The extract was taken to dryness and the residue was dissolved in chloroform containing diosgenin which was initially present in bound form.

Purifiction of diosgenin by TLC

The chloroform part containing crude diosgenin was streaked on silica gel G and run in chloroform : acetone (3:1). The position of diosgenin on the plate was determined with the help of ;marker diosgenin. Silica gel powder was scraped off from the position of authentic diosgenin and was treated with glacial acetic acid it was slightly warmed and filtered. The filtrate was used for quantitative determination of diosgenin.

Determination of absorption maxima for reaction mixture

For determination of the absorption maxima, glacial acetic acid, Resorcinol (10,000 ppm) and conc. H_2SO_4 were used. Resorcinol solution was prepared after mixing 100 mg of resorcinol with 10 ml. of glacial acetic acid. 1 mg. of authentic diosgenin was taken in a test tube and dissolved in 1 ml. of glacial acetic acid. To this reaction mixture 1 ml. of resorcinol (10,000 ppm) and 0.2 ml. of conc. H_2SO_4 were added for the development of colouration to light pink. The solution of reaction mixture was allowed to stand for 15-20 minutes. With ;this coloured solution O.D. values at different wave length (n.m.) were determined with the help of spectro colorimeter (Systronics). The O.D. values of diosgenin at different wave length were plotted on a graph paper. Absorption maxima of ;diosgenin was determined.

Preparation of different grades of diosgenin

For the preparation of standard curve, the different grades of diosgenin solutions were prepared. 5 mg. of diosgenin was dissolved in 5 ml. of Glacial acetic acid to make 1,000 ppm. solution. From the stock solution a series of dilute solutions of 900, 800, 700, 600, 500, 400, 300, 200 and 100 ppm. were prepared after dilution with glacial acetic acid.

Preparation of stadnard curve

To each 1 ml. solution of diosgenin, 1 ml. Acetic resorcinol (10,000 ppm) and 2 ml. conc. H_2SO_4 were added for the development of pink colouration having an absorption maxima of 510 n.m. The reaction mixtures were kept at room temperature (27°C) for 15-20 minutes. Then the O.D. values of all solutions were determined with the help of colorimeter and standard curve was prepared.

Table 24 : Production of free and bound form of diosgenin in the yam of different species of *Dioscorea* in the ecological condition of Darjeeling and Sikkim Himalayas.

Species	Free forms of Diosgenin %	Bound form of diosgenin %	Total diasgenin %
D. alata	—	0.07	0.07
D. kamoonensis	0.20	0.28	0.48
D. arachidna	0.30	0.35	0.65
D. sikkimensis	0.25	0.35	0.60
D. bulbifora	0.06	0.74	0.80
D. esculenta	—	0.06	0.06
D. sativum	0.40	1.00	1.40
D. prazeri	0.60	1.60	2.2

Results and Discussion

Various ;methods applied so far in connection with the estimation of diosgenin are observed to involve gravimeteic determination (Selveraj, 1971) and Gas liquid chromatography (Glyzine, 1981). Though densitometric method using TLC seanner (Gunawan et al, 1994) has also been applied, but all these methods are cumbersome and sometimes involve costly machinary not available in all the Laboratories. Pasesnichenko et al (1978) utilised colorimetric method involving conc. H_2SO_4 and 1% formaldehyde to estimate glycoside of diosgenin and not as free diosgenin. Crude diosgenin after extraction is always associated with various other impurities. It has been observed that purification of diosgenin during estimation have not been taken into consideration in most of the cases. During isolation of diosgenin from bound form, acid hydrolysis of the tissue is the must. But during the treatment a considerable amount of diosgenin is lost due to conversion of diosgenin to its diene form which is generally considered waste in pharmaceutical industry (Harborne, 1973). Thus an attempt has been made to work out an easy and

rapid colorimetric method for quantitative estimation of diosgenin after being purified following the method of chromatography.

The procedure which has been worked out for quantitative determination of diosgenin is based on the principle of Bell and Briggs (1942) who noted that some steroidal compounds when treated with resorcinol in acetic acid followed by H_2SO_4 treatment produced characteristic colour. He utilised chemical test in connection with the detection of cholesterol. The proposed method is supposed to be a new and easy one in comparison to those mentioned earlier. More over in this method purification of diosgenin by TLC has been stressed. The colour complex was determined to have absorption maxima at 510 nm (fig.40) and the colour was stable after standing the mixture for 20 minutes and continued to last for 40 minutes. Diosgenin was calculated from the prepared standard curve ranging from 100 ppm to 1000 ppm of solution which was observed to obey Beer's law (Fig.41) the propsed method is supposed to be advantageous because of the fact that diosgenin can be determined from a low concentration of 100 ppm solution. Moreover with the help of this methods only a few milligramme of dried plant material was observed to be sufficient for estimation of diosgenin and it is observed to take a very small duration of time for the estimation of diosgenin dealing with large number of samples.

Most of the authors estimated diosgenin being isolated after acid hydrolosis of the yam of *Dioscorea*. As a result the diosgenin content reported earlier represented the total of free and bound form of diosgenin in the material studied. The proposed method has the advantage to estimate free and bound form of diosgenin and due to involvement of purification of diosgnin by TLC, it is expected to give more accurate result as compared to most of the methods involving estimation gravimetrically. The toble 23 shows the percentage yield of free as well as of bound form of diasgenin in the yam of different species of *Diascoreas* collected from natural habitat condition of Darjeeling and Sikkim Himalayas. It is very intwistifognot that the *D. alala* and *D. esculenta* which are general utilised by the local people as food does not contain the free diasgenin as compared to others i.e. *D. kamoonenis*

(0.20%), *D. arachidna* (0.30%) *D. sikkimensis* (0.25), *D. bulbifera* (0.06%) *D. sativum* (0.40%) and *D. prazeri* (0.60%). Otherwise the total value for some of the species of *Dioscorea* (Table 23) of diosgenin are slightly less than those observed (Table 14) after following the method of Bammi and Randhawa (1975). This is probably due to purification of diosgenin by TLC.

SUMMARY

A new and rapid colorimetric method has been worked out on the basis of development of colour when diosgenin is treated with resorcinol in acetic acid followed by conc. H_2SO_4.

Free form of diosgenin has been isolated when dried powder of yam of *Dioscorea* sp. has been refluxed in presence of chloroform for 15-20 mins. Bound form of diosgenin is obtained when powdered yam is subjected to acid hydrolysis, neutralised and extracted with pet. ether to obtain bound form of diosgenin.

The reaction mixture, consists of 1 ml. of diosgenin dissolved in glacial acetic acid, 1 ml. of resorcinol (10,000 ppm) and 0.2 ml. of conc. H_2SO_4. The solution of reaction mixture is allowed to stand for 15-20 min.

The absorption maxima has been noted to be 510 nm. The standard curve from 100 to 1,000 ppm. of diosgenin obey the Beer'slaw.

Stability of colour of reaction mixture remaines stable for forty mins. at room temperature (27^0C).

In connection with purification of diosgenin TLC has been applied in chloroform and acetone mixture (3:1 v/v).

The proposed method may be considered as an easy and rapid one and with the help of it, the free and bound form of diosgenin has been estimated in each of the yam of eight species.

Only *D.alata* and *D.esculenta* have been noted not to contain any free form of diosgenin but maximum amount of bound form of diosgenin on dry weight basis have been observed in this yam of *D.sativum* (1.00%) and *D. prazeri* (1.60%) growing in Darjeeling and Sikkim Himalayas.

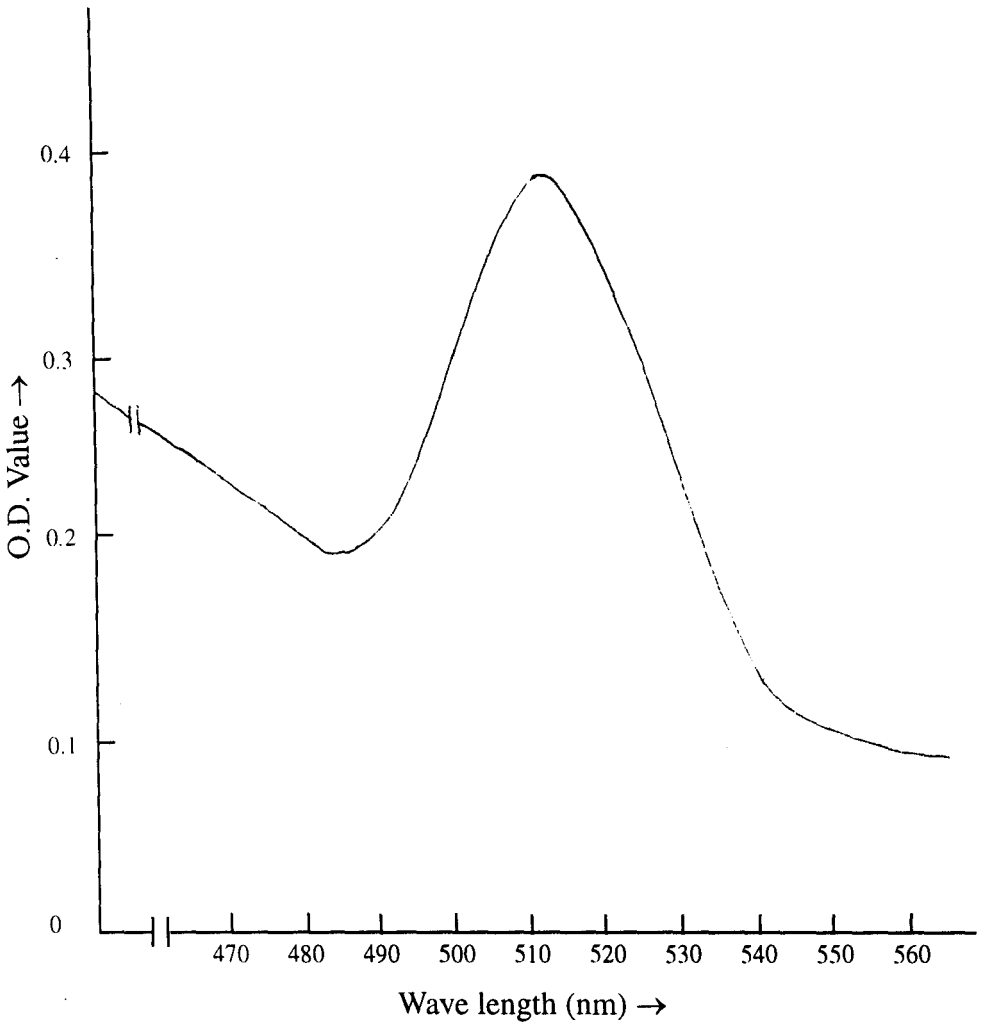

Fig. 40 : Absorption Maxima of Diosgenin (λ max 510 nm)

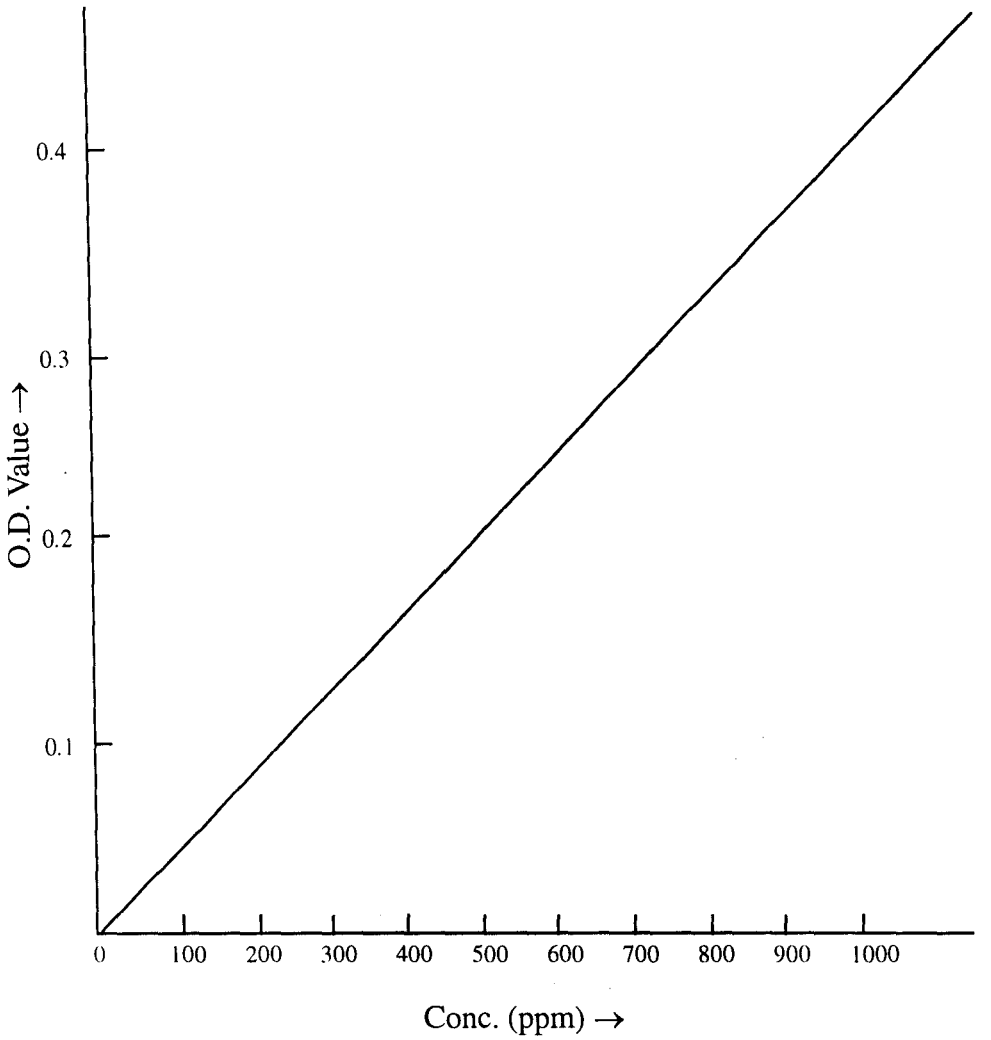

Fig. 41 : Standard curve of Diosgenin

CHAPTER - V

ISOLATION AND CHARACTERISATION OF ANTIFUNGAL CONSTITUENT IN THE YAM OF CULTIVATED SPECIES OF *Dioscorea alata* L.

Introduction

While working on isolation of steroidal constituents from the yam of different species of *Dioscorea* it was observed that even in rainy season, the water extract of the yam of *D. alata* did not show any contamination of micro organism though other extracted material kept in a container showed severe contamination with a fungal strain. The fungus was identified as *Aspergillus niger.* No report is available in connection with the antifungal activity of water extract of *D. alata* with special emphasis on characterisation of chemical constituent.

Recently pthenanthrene derivative from the tuber of *D. delicata* has been ovserved to show antifungal activity against *Ciadosporium cladosporoides* (Haragachi et at. 1999). Hu *et. al.* (1999) studied bioactivity of traditional Chinese herval medicine, *D. composita* against pyricularia oryzae.

In order to understand the nature of chemical inhibitar in the yam of *D. alata* to serve as antifungal agent, investigation has been carried out following conventional phytochemical method.

Materials and Methods

Materials: Yam of *D. alata.*

Methods :

Collection of preparation yam of *D. alata*

Yam of *D. alata* was taken out from the soil and washed in water to free it from soil debris. 500 gms of freshly cut yam was crushed in an electrically operated mixer to form a paste.

Extraction and purification of isolated product (Brian *et al.* 1968)

The pasted material was mixed with 250 ml water and boiled at 100°C for one hour under reflux condition. It was filtered to obtain water extract of the yam. The extract was concentrated and evaporated more or less to dryness. The solid mass left was treated with methanol and refluxed for 30 min. It was filtered to obtain methanolic extract. The methanol extract was concentrated to small volume. Chloroform was added dropwise to produce turbidity in the solution. It was kept overnight in cold condition to obtain crystals.

Thin layer chromatography and Paper cromatography (PC) for identification of the isolated product

Petroleum ether, (b) Butanol : acetic acid : water (4 : 1 : 5, lower fraction) and Methanol : Chloroform (9 : 1), d) phenol saturated with water have been used during paper chromatography. Chloroform : acetone (4 : 1, V/V) and Hexane : acetone (4 : 1, V/V) have been used for TLC.

The isolated product was run with the authentic sample of saponin. The dried paper and TLC plate were placed in iodine chamber to locate the position of the spot for determination of the Rf value.

Preparation of P.D.A.

Composition :	Peeled potato	-	40.00 gm.
	Dextrose	-	2.00 gm
	Agar-agar	-	2.00 gm
	Distilled water	-	100 ml

Small blocks were made from peeled potatoes. All ingredients were weighed according to the composition. Potato blocks were taken in a conical flask placed on a heater to get decoction. It was boiled till the smell of boiled potatoes came out. The resultant decoction was filtered out using strainer. To the filtered solution dextrose was added and stirred well until the dextrose dissolved. Finally the agar was added and again heated on a water bath to melt the agar completely. The medium was poured in sterile culture tubes and immediately plugged using sterile cotton. For slant preparation 5-6 ml of medium was poured

where as for stab preparation 20 ml of medium was poured in each culture tubes. All culture tubes were placed in an autoclave to make it free from contamination at 120°C, 15 lb pressure for 15 min. All culture tubes were taken out of the autoclave. Stabs were placed in a test tube stand in a vertical position and slants were placed in a slanting position.

Inocculation of *A. niger* in to culture medium

Two types of slants were used. In one type *A. niger* was inocculated to freshly prepared P.D.A. in the test tube. In another tube the culture medium was previously mixed with 100 ppm of the isolated product in water and the tube was inocculated with the same fungus. The observations were noted after seven days, keeping both the culture tubes at room temperature (25°C).

Result and Discussion :

The crystals that were obtained after extraction of yam of *D. alata* with water and subsequent Methanol chloroform treatment, were filtered off and recrystallised from methanol chloroform mixture, when 30 mg of crystals having m.p. 287°C was obtained. That the isolated product was a saponin was confirmed by the produciton of heavy frothing while boiling the crystals dissolved in water.

Table 25 :Paper chromatographic behaviour of Dioscin and the saponin like isolated natural product inthe water extract of the yam of *D. alata.*

Chemicals	**Petroleum ether Rf.**	**Butanol : Acetic acid : water (4:1:5 V/V/V) Rf.**	**Methanol : Chloroform (9 : 1,V/V) Rf.**
Dioscin	0	0.25	0.45
Saponin like isolated natural product	0	0.25	0.45

The IR spectrum of the isolated saponin showed characteristic peaks λmax 3350 (Broad) 1640, 1375, 1175, 1050, 850, 821, 720 cm^{-1} (Fig. 42) and which are superimpossible with those of authentic dioscin. It was further confirmed

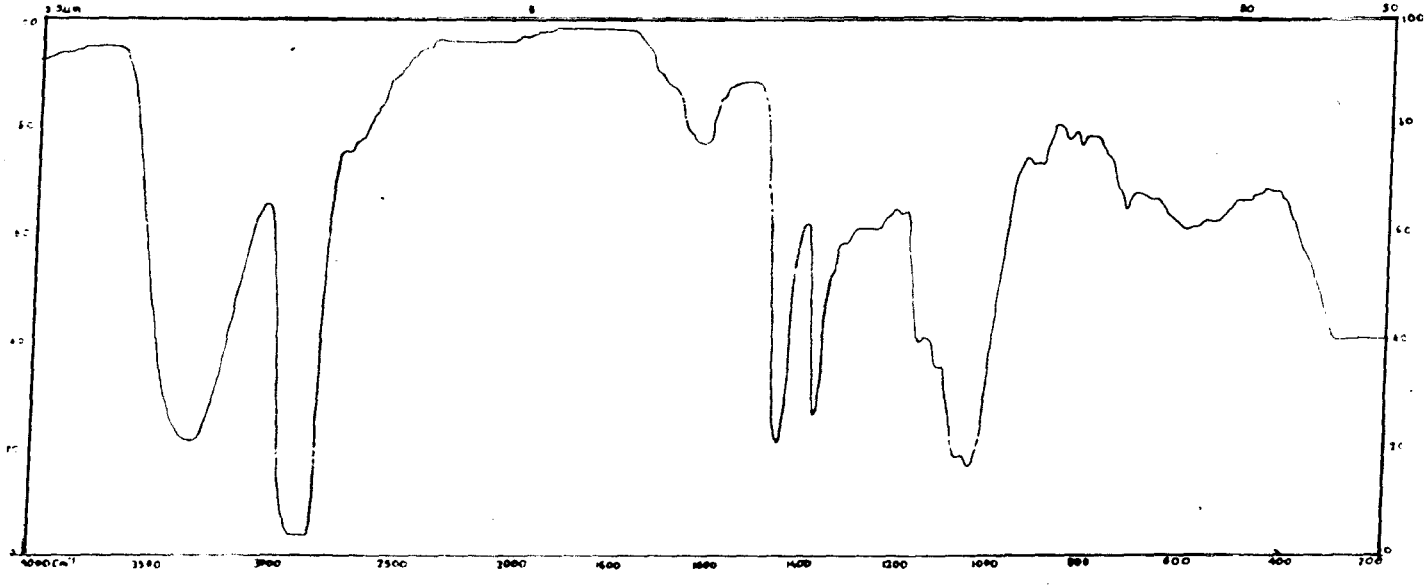

Fig. 42. IR spectrum of dioscin.

with the chromatographic behaviour of the isolated saponin with that of Dioscin (Table 25). The sapogenin was derived after following the procedure adopted by Brain *et al.* (1968). The residue was hydrolysed with molar HCl for 6 hrs. It was neutralised and was shaken with chloroform. The chloroform part was evaporated to dry mess and fine crystals were obtained from chloroform, methanol mixture. The crystal showed m.p. 206-208°C. It was subjected Rf. 0.58, Hexane : Acetone (4 : 1, V/V) Rf. 0.32. The plate showed purple spots after spraying with Antimony chloride in conc. HCl and subsequent heating at 110°C for 10 min. The physical and chemical properties including IR spectrum analysis were the same with those obtained in connection with the authentic diosgenin (Fig. 39) and that has been represented in the chapter dealing with pharmacognosy work.

After removal of diosgenin from the acid hydrolysed product of saponin, the filtrate was neutralised with barium carbonate. After filtration the filtrate was concentrated under reduced pressure and spotted on paper for chromatography in solvent mixture of Butanol : acetic acid : water (4:1:5V/V/V, upper layer) and phenol saturated with water, sugars from the hydrolysed product were identified to be glucose and Rhammose Rfs of different sugars in various solvent mixture during paper chromatography have been represented in the table26.

Table 26.: Identification of sugars after acid hydrolysis of saponin by paper chromatography.

Chemicals	**Butanol : acetic acid : water (4:1:5, V/V/V) Rf.**	**Phenol Saturated with water Rf.**	**Colour of the spot due to aniline hydrogen pthalate.**
Sugars obtained from acid hydrolysed saponin			
Sugar I	0.13	0.24	Brown
Sugar II	0.34	0.59	Yellow brown
Authentic			
Glucose	0.13	0.34	Brown
Rham nose	0.34	0.59	Yellow brown

Fig. 43 A shows that the slant without dioscin of 100 ppm served medium for good growth of *A. niger* as reflected fromt he very good formation of white mat of mycellium. Whereas Fig. 43 B shows the dead mycellium which became black due to degeneration of hyphal mat due to lysis caused by dioscin (100 ppm) in the medium. It is very interesting to note that the centrally placed small part of agar without dioscin to serve inoculum shows white mat of hyphae still in living condition. Antifungal activity of dioscin against *A. niger* has not been reported earlier though Roddick *et al.* (1990) showed antifungal activity of solamargine. While working with antimicrobial tests of natural product of plant origin Imai *et al.* (1967) mentioned that *D. tokoro* gave dioscin as active agent. Recently Haraguchi *et al.* (1999) has isolated a phenanthrene derivative from *D. delicata* showing antifungal activity against *Cladosporium cladospodoides*. Solamargine may be considered as the nitrogen analogue of rhamnose-glucose-rhanmose bearing Dioscin (Fig. 44). Because both of them have got the same sugar moiety i.e. rhamnose, glucose-ramnose being attached to solasodine, a steroidal alkaloid to form solamargine and also to diosgenin, a sapogenin to form dioscin. As the nitrogen in the chemical structure of solasodine is replaced by oxygen in diosgenin, the former is considered nitrogen analogue of the latter. According to Roddick et al. (1990) rhamnose-glucose-rhamnose in solamargine cause significant disruption of membrane in biological materials due to lysis. It is expectedthat the same type of bioactivity may be claimed to be due to rhamnose-glucose-rhamnose in Dioscin having the same structural configuration as that of solamargine. The accumulation of dioscin in the yam of *D. alata* may be claimed to help the underground part of the plant to become free from the attack of microbes.

SUMMARY

The water extract of the yam of *Dioscorea alata* has been observed to contain a chemical constituent having the antifungal property against *Aspergillus niger*.

After purification following conventional method of fractional crystallisation from methanol chloroform mixture, crystals having m.p. 287°C has been obtained.

Following comparative behaviour and I.R. spectrum of isolated product with those of authentic sample, it was identified as dioscin, a saponin. The glycoside nature of it has been verified after identification of diosgenin and the sugar components of rhammose and glucose, during acid hydrolysis of saponin.

The antifungal activity of dioscin against *A. niger* has been verified during the culture of the fungus in P.D.A. medium mixed with dioscin as compared to the control showing good growth of the fungus in a medium free from dioscin.

The antifungal activition of dioscin is being claimed to be due to chacotriose i.e. rhamnos-glucose-rhamnose combination of sugar in dioscin.

CHAPTER - VI

SURVEY ON PRESENT DAY ETHNIC USE OF DIFFERENT SPECIES OF *Dioscorea* IN DARJEELING AND SIKKIM HIMALAYAS WITH SPECIAL INTEREST ON DIFFERENT HERBAL SYSTEMS OF MEDICINE AVAILABLE IN THE REGION

Introduction

The knowledge of herbal medicine that has come through generation verbally is the main subject of ethnobotany. The term "Ethnobotany" was first used by Harshberger (1885) and its scope was much elaborated later (Ford, 1978; Faulks 1958) Since then there has been a growing interest in the field (Jain 1986; Martin 1995) and has received much attention in certain parts of the world particularly in the underdeveloped and or developing countries where small or large proportion of population still depend on natural resources in particularly indigenous condition and the impact of modern system of medicine has not reached them. This subject assume great importance in enhancing our knowledge about plants grown and used by native tribal communities. There is no doubt that ethnobotany is a multidisciplinary science. In its totality the subject involve anthropology, sociology, botany and of course medicinal and economic botany (Jain 1981).

Botanically, the Darjeeling and Sikkim Himalayas may be said to be the richest source of medicinal and aromatic plants in India. Amongst the diverse floristic elements many plants are of religious, social and medicinal value. Their usage through the course of countless generations have rendered them to become indispensable part of culture of the people of the region, a sizeable population being comprised of the tribals.

The exhaustive floristic work in Darjeeling and Sikkim Himalayas was made by Sir J.D.Hooker during 1871-97, but he did not lay much stress on the healing properties of the indigenous medicinal plants. The most comprehensive work on medicinal plants in the region had to wait for many years (Biswas and

Chopra 1940; Biswas 1956). Later observation on ethnobotanical studies in the region have been published (Bennet 1983; Tamang and Yonzone 1982; Yonzone et al 1884, 1985). But all the authors so far attempted in this line of work, did not mention *Dioscorea* as traditional or tribal medicines in the region. Various ehtno medicinal use of different species of *Dioscorea* has been reported from Andhra Pradesh, India (Reddy et al 1998), Japan (Nakamura et at 1998) and Australia (Min et al 1998) but proper identification of the natural products responsible for their remedial activity has not been worked out

Recently Mulleng et al (1999) isolated linamarin, a toxic constituent for human and animal health from some economically important yam of *Dioscorea species*.

Since the discovery that diosgenin - a sapogenin could be used as a precursor for the synthesis of cortisone, progesterone and C21 steroidal oral contraseptives, much attention was given only on a limited number of *Dioscorea sp.* disregarding further investigation on purposeful utilisation of wild and traditionally important species of *Dioscorea.*

From survey it appears that though modern system of medicine is available in the region, different species of *Dioscorea* are still being used by different tribal communities as ethnomedicine and various purposes. Besides it has been observed that a number of systems of herbal medicine are still in vogue in the region.

This paper deals with a discussion on present day use of different species of *Dioscorea* by the local as well as tribal people with special emphasise on the past and present day status of different herbal systems of medicine in the region.

Material and Methods

Materials : Eight different species of *Dioscorea* have been taken into consideration. These are : *D.alata* L. (Ghartarul), *D.kamoonensis* Kunth (Bhyagur), *D. arachindna* (Bharlang) Prain & Burkill, *D.sikkimensis*, Prain & Burkill (Niltarul), *D. bulbifera* L.(Bontarul), *D. esculenta* Prain & Burkill, (Sutuni) *D. prazeri,* Prain & Burkill (Cucurtarul) and *D. sativum* L. (Githa).

Methods

Field Work

Intensive field work has been done among 15 different types of tribal population i.e. Lepchas, Sherpus, Bhutias, Kami. Dorji, Sarki, Sunar, Manager, Gurung, Ghate, Chettri, Bahum, Thakuri, Limbus and Rais available at fifteen different villages in Darjeeling district such as Tanek, Somalbung, Singi, Garubathan, Mansong, Khari, Samthar, Nangsonden, Lolley, Gitdebling, Shhibo, Suruke, Sindebling, Bunbusty, Bijanbari and seven villages in Sikkim i.e. Kerbari, Dentam, Ratomte, Singtam, Jorthang, Namchi, Rambhabung have been visited. Information on ethnomedicinal value of plants in the region has been collected from knowledgeable persons, medicine men and practisioner.

Literature

Some published and unpublished literature have been consulted for gathering information.

Herberia

Plants and their parts were collected and herbarium sheets have been prepared. these have been identified after comparing with those of authentic specimens inthe Central National Herbarium, Botanical Survey of India, Hawrah, West Bengal.

Results and Discussion

It is established that majority of *Dioscorea sp.* are distributed in tropical and subtropical region all over the world including India. During survey eight different species of *Doscorea* have been observed to be distributed mainly in the lower and middle hills in the region. It has also been observed that the region of Darjeeling and Sikkim Himalayas is occupied by the people belonging to different ethnic groups. It is apart from the three majot ethnics – Bhutia, Lepcha and Nepalese, a conglomerate of over 20 ethnic tribes and a still more number of subtribes (Rai and Sharma1996).

The climate in Northern part of Darjeeling and Sikkim Himalayas is dry and cold and most of the land is having with higher altitude which is beyond the range of monsoon resembling arctic region. In Southern part of the region the climate is damp and warm at lower altitude such as is encountered in the tropical region. It is not surprising therefore that distribution of *Dioscoea sp* and thick population of different tribal people are concentrated mostly in such tropical climate.

The Darjeeling and Sikkim Himalayas lie in that part of Asia where Nepal, China, India, Tibet and Bhutan are very close together. Though these areas have distinctive peoples, climates, plants and animals but types appear in common and tend to intermingle occasionally where the boundaries of different great areas merge together. The region under study is roughly bounded by Nepal on the westflank, China-Tibet on the North and major part of the East. A part of the East is bounded by Bhutan and West bengal is on the South.

From time immemorial the region was very much influenced by the Lepcha's wonderful knowledge of the medicinal plants in the region (Biswas and Chopra 1940). The land form was inhabited by the Lepchas for a greater part of the history and Lepcha system of herbal medicine was predominated for a long time.

The accession of Raja Phuntsong Namgyal in 1641 A D was a landmark event in the Sikimese history and a starting point of the reign of Bhutia King in Sikkim and the advent of Buddhism. Due to revages of war which was almost regular activity during the 17th century, Sikkim witnessed a repeated transformation over its boundaries. In 1706 what is now the Kalimpong subdivision of present Darjeeling district was taken from the Raja of Sikkim by the King of Bhutan. Until 1865 the area of Kalimpong which was the territory of Bhutan was annexed to India by the British India. During the period the Lepcha system of practice in medicinal plants was mixed up with the culture of Bhutanese, The Raja of Sikkim later became engaged in unsuccessful struggle with the Gurkhas who had invaded Sikkim in 1780. During the next 30 years they over ran Sikkim as far East as the Teesta. In the mean time war broke out between the East India

Company and the Nepalese at the end of which in 1817 by the treaty of "Titaliya" the part which the Nepalese wrested from the Raja of Sikkim was ceded to the Company (Economic Development Profile, 1978). It became obvious therefore that Nepali system of herbal medicine was introduced in the region. At that time the East India Company restored whole of the country between the Mechi and Tista to the Raja and guaranteed his sovereignty. Sikkim was them maintained as a buffer state between Nepal and Bhutan. Lord Willium Bentink the then Governor General expressed his desire to possess the hill of Darjeeling on account of cool climate for the purpose of enabling the servant of his government suffering from sickness. The then Raja of Sikkim out of friendship for the said governot general presented Darjeeling in 1833 to the East India Company that is all the land South to the Great Rangit river, west of Rongo and Mahanadi river. At that time Darjeeling was inaccessible tract of forest. An excellent sanatarium was established for troops and others. European houses were built up with rapid development of township and European systems of medicine was introduced in Darjeeling. In the meantime relation between the Raja of Sikkim and East India Company deteriorated. In 1850 annexation was brought about so that the British territory in Darjeeling became continuous with British India. As a result European system of treatment of disease was reaching gradually even upto the interior of the hills of Darjeeling and Kalimpong of British India and remaining part of Sikkim was very much dependent on primitive system of treatment of diseases practised in the past by the medicine men, herbalists of different hill tribes in the region.

After the independence and the birth of Indian republic, the hills of Darjeeling was converted into Darjeeling district of the State of West Bengal and Sikkim was merged with the Indian Union as the 22nd state in the year 1975.

In Sikkim majority of the population (70%) at present are from Nepali stock. The Bhutia and Lepchas constitute about 30% of the population. The original inhabitant in Darjeeling district was the Lepchas which were rapidly outnumbered by settlers from Nepal and Sikkim. Once the majority of the non-Indian nationals of Darjeeling was Tibetans (Economic Development Profile 1978).

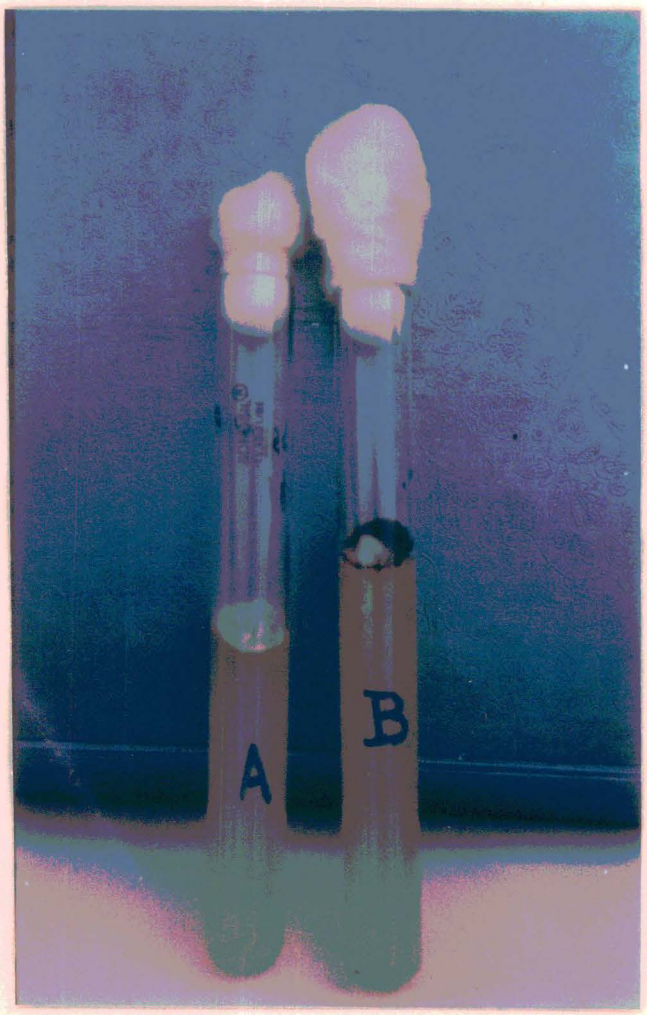

Fig. 43 Antifungal activity of dioscin against Aspergillus niger.
(A). Slant without dioscin showing good growth of fungal hyphae.
(B). Slant with dioscin showing black mass of lytic hyphae surrounding dioscin free agar bearing in occulum.

From survey it appears that at present there are four different systems of traditional medicines available in the region - such as Lepchas, Nepalese, Tibetan and Bhutia system. Though Rai and Sharma (1996) mentioned the availability of three systems of medicine but they did not mention the Bhutia system, though this system is being cultured at different places in the region.

Deeply seated in its antiquity but very thinly doccumented the Nepali system of herbal medicine survives to day in the region as "Jaributy" or just semply as "Pahaday Dabai". The practise of herbal medicine has in its true nature not yet attained a system status in itself as in the case with Tibetan system. As because it is not yet organised set up. The "Jaributi" system has to bring up the several disjoined systems together to get proper status (Rai and Sharma 1996). From the survey it appears that Tibetan medicinal prescription usually comes in the form of powder and different forms of extract much in contrast to Nepali medicine where the prescription normally is unprocessed plant product and comes in all of its natural shape, size, colour and form such as bark, twigs, fruits leaves, seeds etc. representing traditional medicine. Though the Tibetan system had its root in ancient Tibet where spirituality dominated in practise but the general practitioner of Tibetan medicine at present relies more on the plant than spirituality.

Out of eight different species six species of *Dioscorea* such as yams and bulbils of *D. alata, D. kamoonensis, D. sikkimensis, D. bulbifera, D. esculenta* and *D. sativum* are edible (table 27) but *D. arachidna* and *D. prazeri* are nonedible types. It has also been observed that though *D. sativum* is considered edible but local people are not habituated with the regular use of the plant though the Lepchas and Bhutias use it regularly. Besides the mature yam of *D. kamoonensis* used by the tribal people is also given to pigs as feed after boiling in water. In connection with the use of *D.esculenta* it has been observed that though local people eat yams but only the Lepchas generally cultivate the species because the local people strongly believe that if the higher castes other than Lepchas cultivate it, generation of the cultivator may degenerate. It is also observed that Nepali people do not eat all the edible *Dioscoreas* regularly but they eat specially the yam of *D. alata, D.sikkimensis, D. bulbifera* and *D. esculenta* to celebrate the festival of "Makar Sankranti" in the month of January in each year. (Fig. 28).

Table 27. :Ethnobotanical use of *Dioscorea sp.* as food and vegetable in Darjeeling and Sikkim Himalayas

Plant species	Local name	Locality	Plant part	User/tribe	Preparation of edible part
D. alata L.	Khamalu	All localities	Rhizome Bulbil	Local people including tribals	Rhizone/bulbil cleaned thoroughly boiled in water cooked or eaten as vegetable.
			Rhizome	Poor people including tribals	Rhizome cleaned thoroughly boiled in water to make it a paste and use as alternative to rice.

Table 28 : Ethnoreligions use of *Dioscorea* sp. by Nepalese in Darjeeling and Sikkim HImalayas

Plant species	Local name	Locality	Plant part	User	Mode of use
D. alata L.	Khamalu	All localities	Rhizome	Nepalese	All Nepali peiopl rhizome boiled to celebrate the festival of "Makear Sankranti" in the month of January each year
D. sikkimensis Prain & Burkil	Niltarul	Do	Do	Do	Do
D. bulbifera L.	Bantarul	Do	Do	Do	Do
D. esculenta	Sutuni	Do	Do	Do	Do

Tubers of certain species of *Dioscorea* are used as staple food in a number of countries of West Africa, South America and the Indian sub-continent. Species like *D. alata, D. esculenta, D. batatas* and *D. bulbifera* fall under this category.

The edible species particularly *D. alata* and *D. esculenta* are characterised by very high tuber yield. The chemical composition in the tuber of the edible types, particularly their nutritional values has been studied earlier (Coursey, 1967; Martin and Thompson, 1971). The starch content in the tuber has been observed to range from 0.42 to 0.78 percent in the three saponin bearing species i.e. *D. composita, D. floribunda* and *D. deltoidea.* However in the edible species, the starch constituted the major proportion of the tuber. It was observed to become 10.59 percent in *D. alata* and 11.53 percent in *D. esculenta* on fresh weight basis. Besides it was also observed that *D. alata* had the least amount of total sugar (1.37 percent) and *D. esculenta* also had more sucrose and glucose than other species. *D. alata* had the lowest amount of sucrose. No distinct pattern of distribution of sugar was discernible to contrast the two groups of species. Crude protein int he tuber was lower in Saponin bearing species (0.96 to 0.426 percent) than in the edible species (2.178 to 2.693 percent). The soluble amino acid pattern was similar in all species but the quantity of individual aminoacid in the tuber varied considerably between different species.

In general saponin bearing *Dioscorea sp.* contained less nitrogen in the tuber than that of edible species, however they had more phosphorous and potassium. There was little difference in iron content in the tuber of different species. According to Bammi and Randhawa (1976) total yields of sterol in the tuber of *D. alata* and *D. esculenta* are 3.37 mg/100g and 0.92 mg/100g respectively as compared to high sterol content of 9.72 mg/100g and 4.68 mg/ 100g in commercially important non edible diosgenin yielding plant like *D. composita* and *D. floribunda* respectively.

Worshipping for blessings and betterment of life is a practise common to all human beings. During worshipping traditional rituals are being performed. SInce time immemorial these customs are being observed in tribal communities in Darjeeling and Sikkim Himalayas. But there is no doubt that from ancient days only those plants are having with economic benefits they are being treated as sacred plants.

One of the primitive traits in the culture of the people of Darjeeling and Sikkim Himalayas is the existence of spiritualists known as "Jhankri" was also practice herbalism. In connection with ethnospiritual use of *Dioscorea* (Table 29) it has been observed that rhizome of *D. arachidna* is used by

Phedangmas (Limbus' Doctor) and Bizuwas (Rais' Doctor) to kill evil spirit. Common people believe that persons affected with ghost or evil spirit are having with less energy

Table 29. : Ethnospiritual use of different species of *Dioscorea* in Darjeeling - Sikkim Himalayas

Plant Species	Local Name	Locality	Plant part	User/tribe	Purpose/ mode of use
D. arachidna Prain & Burkill	Bharlang	Tanek, Chibo, Suruk, Bung-busty, Sinder-bung, Samthar	Rhizome	Limbus, Rais	Fresh rhizome after being cleared are eaten by the tribal people as per recomendation of Phedangmas (Limbus' doctor and Bizuwas (Rais' doctor) commonly known as "Jhankri" the Spiritualists in the region.

and vigour. It is expected that the yam of *D. arachidna* may contain some natural product having stimulatory property and which may be called mind altering substance.

From literature it appears that during fifteenth century the Europeans were habituated using a few mind altering substances. At that time the population had no coffee, tea, opium, cocaine, tobacco etc. It has been reported by Lewis and Elvin-Lewis (1977) that substitute like roasted parts of yam of *Dioscorea* was used as stimulating beverage. They also mentioned that tea made from the roots of *D. villosa* was taken by Meskwakis to relieve body pain in fatigue condition. One can recall the ritualistic use of "Soma" or "Somrash" of the Hindu epics. About 20 different plants have been attributed to this name (Jain 1981). According to Shah and Badola (1977) climber like *D. bulbifera* has got certain similarity with that of "Soma", representing ancient literature.

As regards the ethnomedicinal use (Table 30) of different species of *Dioscorea* the Lepchas in the region of Darjeeling and Sikkim Himalayas generally use the yam of *D. prazeri* for hair washing to kill lice and fish poisoning, but others like Limbus, Rais, Ramang, Chettris and Mongers use the water docoction of the yam of the species for curing wound of human being. They also use the powder of the yam to check population by induction of abortion. It has

been observed that Nepali people use the yam of *D. bulbifera* for remedy against piles, bronchities, tumors, asthma, dysentry. Besides they also use it as anthelmintic and to relieve pain in the abdomen. Lee *et al.* (1999) observed anti-diabetic activity of *D.batatas* in mice. Min et al. (1998) carried out ethnopharmacological investigation, taking rabbit model to show that the root tuber of *D. opposita* had regulative effect on the thyroid disfunction of thyroidectomized animals.

Table - 30 : Ethnomedicinal use of different species of *Dioscorea* in Darjeeling and Sikkim Himalays.

Plant Species	Local Name	Locality	Plant part	User/tribe	Preparation/ Dosage	Purpose
D. sativum L.	Githa	Gorubathan, Mansong, Tanak, Bung-busty, Bijan-bari, Surat, Santhar.	Rhizome	All tribal people	Rhizome boiled in	To kill worms present in stomach to check gastric trouble
D. prajeri Prain & Burkill	Cucur-tarul	Nangsondang, Tanak, Chhibo, Khani, Suruk, Gitedebling	Rhizome	All tribal people	Crushed rhizome boiled in water	Water solution of rhizome used allover the surrounding of wound for cheking swelling and infection.
			Rhizome	Lepchas	Crushed boiled in water.	Hair washing for killing lice also used as fish poison.
		Tunuk, Jorthang, Ratomale	Rhizome	Limbus, Rais, Tamang, Chettris, Mongers	Powder of dried rhizome	For abortion
D. bulbifera L	Bantarul	All localities	Rhizome	Nepali people	Powder of dried rhizome	Piles, Asthma Dysentry, Bronchitic, Tumors, Abdominal pain.

Seventy seven species representing 71 genera and 42 families of flowering plants used as crude drugs in veternary practices by folklore in Warangal district

of Andhra Pradesh, India have been enumerated (Reddy et al. 1998). Out of these plants, *D. bulbifera* and *D. pentaphylla* are commonly used by the local people. Though they mentioned common veternary diseases of the district such as anthrax, cough, dysentry, inflammatory, diseases, lack of milk secretion etc. but no information with special emphasis on scientific evaluation of the species of *Dioscorea* as veternary medicine is available.

Ethnoveternary use of different species of *Dioscorea* (Table - 31) has been observed in the region of Darjeeling and Sikkim Himalayas. The yam of *D. kamoonensis* is commonly used as a remedy against "Bhyagute" disease of cattle by all the tribal people specially the Lepchas, Bhutias and Sherpus in the region. This type of disease has a symptoms of respiratory trouble of cattle producing irritating sound. Certain type of slimy fluid generally comes out as tears from eyes. Milk producing cattle generally stop to give milk. Body temperature rises to 104° - 106°F. the surrounding areas of head, neck, chest, excretary and reproductive organs swell up. Blood comes out with the stool. Generally animal dies within 12-24 hours after the initiation of the symptoms. For remedy against the disease yams of *D. kamoonensis* are cut into pieces and are forced to enter into the mouth of the cattle as feed. This is generally repeated thrice daily for one week. The scientific name of the Bhyagute disease is known as "Haemorrhagia Septimae". The name of the causal organism of the disease is *Pasteurella bovisentica*. In modern therapy Sulphamezithin (5 gm.) and vaccination is used. The rhizome of *D. sativum* has been ovserved to become effective against parasitic disease caused by *Fasciola hepatica* in cattle and for which the animal becomes weak and their eyes look yellow with swelling of stomach. Yams of *D. sativum* are generally given to the diseased cattle to eat the yam after boiling with water that most of the diseases which are caused by different microbes and parasites are cured by the use of yams of different *Dioscorea* and it is expected that all these species must have some natural product having anti microbial and antiparasitic activity and which are yet to be workedout.

The modern antibiotic era can be said to have opened on February 12, 1941 with the first clinical trial of Penicillin. This was followed by the introduction of one after another of major antibiotic substances which remain the mainstay of clinical therapy of infectious disease to this date. Intensive screening has

resulted in literature the description of more than 2000 individual antibiotic (Mitscher 1975).

Table - 31 : Ethnoveternary use of different species of *Dioscorea* in Darjeeling and Sikkim Himalays.

Plant Species	Local Name	Locality	Plant part	User/tribe	Name of the disease & symptoms	Dosage/ Preparation
D.kamoonensis Kunth		Tanek, Singi, Samaltsung, Khari, Samathar, Lolay, Gitdebling, Suruk.	Rhizome	Lepchas, Sherpas, Kam,Dorjee Sarki,Sunar, Manger, Gurung, Ghate, Chettri, Bahun, Thakuri.	"Bhyagute" disease of cattle, the scientific name of which is *Haemorrhagia septimae"* caused by *"Pasteurella boviseptica".* Difficulty in respiration producing some irritating sound, certain type of slippery fluid comes out from the eys, milk producing cattle stop to give milk, body temp. rise to 104°F-106°F, swelling of head, neck, chest, excretory & reproductive organs, stool with blood, usually the animal die within 12 hrs. after infection.	Rhizomes of the plant should be cut into pieces & should supplied into the mouth of the diseased cattle. This should be contained thrice daily for one week Modern therapy Silphamezithin (5gm/day) or vaccination.
D. sativum L	Githa	Tanek, Singi, Khari, Lolay, Gitdebling, Suruk.	Rhizome	Lepchas, Bhutias, Sherpas, Manager, Bahun, Sarki.	Parasitic disease of cattle caused by *"Fasiola hepatica"* Cattle becomes weak and eyes look yellow in colour, swelling of stomach.	Rhizome of the plant should be boiled in water & after boiling the rhizome should be given to the infected cattle to eat.

Man has doubtless been aware at least dimly for centuries that antimicrobial agents are present in higher plants because the folk literature contains frequent references to such uses stretching back for at least 6000 years. Large scale screening programme for antimicrobial agents from higher plants were undertaken in the 1940's using methodology which enables one to judge the relative worth of results. An examination of *Dioscorea bulbifera* from Western Samoa in the Pascific area by Norton et al. (1973) it was found that the plant to be of greatest interest specially in connection with its antimicrobial activity.

While searching for biologically active plant ingradiants by means of antimicrobial tests on oriental crude drug preparations Imai et al. (1967) observed that *D. tokoro* gave Dioscin, Gracillim and Dioscinprosapogenin as active agents. This is followed up by the study of the activity of 23 steroidal sapogenin and saponins which indicated that only saponins are active. According to them well known haemolytic activity of saponins probably prevent these agents from playing a role in systemic infections of humans. Recently a phenanthrene derivative has been isolated from the tuber peels of *D. delicata,* a natural medicine in Japan, to show its antifungal activity against *Cladosporium cladosporoides* (Haraguchi 1999).

Dioscin is a glycoside of diosgenin (Fig. 44) and is commonly present in the yam od *Dioscorea,* structurally it has chacotriose i.e. rhamnose - glucose rhamnose type of carbohydrate moiety linked with diosgenin. Though haemolytic property of Dioscin has not been worked out in details but the lytic property of solamargine which is also chacotriose of solasodine - a nitrogen analogue of diosgenin (Fig. 44), has been worked out in details. Solamargine causes significant disruption of phosphatidyl, cholesterol liposomes (Roddick et al. 1990). Its deleterious effect on variety of structures renging from synthetic membrance (Roddick and Drysdate 1984) through organelles and cells (Roddick 1978) to living organism (Tingey 1984) including man have been worked out. Solamargine has been observed to show lytic effect on *Penicillium notatum* derived protoplast and bovine erythricytes (Roddicket al 1990). Erythrocytes are much more susceptible to lysis. Solamargine caused 100% haemolysis at 20μM. The significance of this phenomenon is generally related to chacortiose nature of carbohydrate.

Recently Puri et al (1999) confirmed the similar structural property of solasodine and diosgenin with the help of H^1 and ^{13}C assignment by two dimensional NMR spectroscopy. According to them chemical shift of protons for both the chemicals are very close.

It is obvious therefore that haemolytic property of Dioscin may be attributed to the presence of rhamnose-glucose-rhamnose combination in the carbohydrate and which is available in different *Dioscorea* showing ethnomedicinal value. However, the biochemical basis of the effect is not yet understood.

Thus the traditional use of elimination of water soluble part of the yam of edible species and utilisation of yam with water soluble part for the cure of wound infection and other type of infection may be claimed to be due to toxic constituent like dioscin in the yam of *Dioscorea* and which has been justified by the lytic effect in connection with antimicrobial activity of dioscin and that has been represented in the chapter V.

Beneytout et al (1995) claimed diosgenin present in *Dioscorea* as a new megakaryocytic differentiation of HEL (Human Erythroleukemia) cells and observed that diosgenin addition to HEL TIB 180 cell line induces morphological and biochemical changes characteristic for megakaryocytic cells assessed by cellular morphology, endomitotic process and glycoprotein content. Erythro leukemia is a disorder that combines involvement of red cell precursor and myeloid precursor to show malignant alteration of several cell lines in the blood.

The extraction, quantification and identification of the cynoglucosides in economically important yam varieties was carried out by Mulleng et al (1999) in different varieties of four *Dioscorea* species namely *D. alata*, *D, cayenensis*, *D. esculenta* and *D. ratundata.* Taking the help of HPLC then confirmed linamarin as the main cyanoglycoside in yams. This result could be of reference in appropriate selectivity in the promotion of desirable cultivars of yam for the food and health industries, since cyanide from linamarin, apart from interfering with oxidative processes of metabolism, also produces pancreatic damage by free radical mechanism reaction and is therefore thought to be a factor in malnutrition related diabetes. Thus the cultivars with low levels of cyanoglucoside should be favoured for consumption and cultivation.

SUMMARY

During the survey on ethnobotanical practice available in the region of Darjeeling and Sikkim Himalayas, it has been observed that four different systems of traditional medicines such as Lepcha, Nepalese, Tibetan and Bhutia. Systems of medicine are still being cultured at different places in the region.

Out of eight species of *Diascorea* yam of six species such as *D. alata, D. kamoonensis, D. sikkimensis, D. bulbifera, D. esculenta* and *D. sativum* are edible but *D. arachidna* and *D. prazeri* are non edible.

Ethnoreligious use of yam of *D. alata, D. sikkimensis, D. bulbifera* and *D. esculenta* has been observed by the Nepali people on the day of "Makar Sankranti".

Ethno spiritual use of yam of *D. arachidna* by Limbus and Rais people has been observed.

In connection with ethno-veternary use, *D. kamoonensis* acts as a remedy against "Bhyagnte" disease (Haemorrhagia septimae)of cattle caused by *Pasteurella bovistca.* The yam of *D. satvum* is being used as a remedy against parasitic disease of cattle caused by *Fasiola hepatica.*

The traditional use that the edible yam of *Diascorea* should be boiled with water and is to be discarded is well justified by the investigation that the boiled water contains toxic factor like dioscin, a saponin having a lytic property to damage red blood cell, or fungal cell due to its Chacotrise sugar moiety containing Rhammnose, Rhammnose and glucose to diasgenin.

However it is expected that the yam of *Dioscorea* containing water soluble part of dioscin becomes effective against microbial infection when it is used external to human or animal body.

CHAPTER - VII

INCREASED PRODUCTION OF FREE DIOSGENIN DUE TO GLYCOSIDASE LIKE ACTIVITY IN THE YAM OF *Dioscorea prazeri* Prain & Burkill. SUBJECTED TO HIGH TEMPERATURE TREATMENT

Introduction

The steroid hormone industry has been fast developing during past few years in India. The raw material used by the industry in India is mainly diosgenin. Diosgenin is generally present in most of the *Dioscorea* sp. in two forms, free diosgenin and glycosides of diosgenin or saponin. (Hardman and Fazli 1972). An easy and rapid colorimetric method has already been established and represented in connection with the quantitative estimation of free diosgenin in the yam of *Dioscorea.* The conventional method of isolation of diosgenin, now-a-days in generally done by acid hyrolysis of raw material. But during acid hydrolysis some amount of diosgenin is converted to diene form which is generally regarded as waste not being used is phytochemical industry. Maximum of thirty percent diene form of diosgenin has been observed to be produced during acid hydrolysis.

From preliminary observation it has been observed that during high temperature treatment of yam of *D. prazeri*, a considerable amount of free diosgenin has been observed to be increased.

It is the purpose of this part of work is to isolate enzyme fraction from the yam of *D. prazeri* subjected to high temperature to understand the nature of activity of spaonin to diosgenin.

Materials and methods

Material : Fresh yam of *D. prazeri.*

Methods

Collection and preparation of yam

Fresh yams were collected and were cleaned with water to remove all the soil particles after clipping the roots. They were then cut into small pieces using a chopping knife.

Treatment of yam with high temperature

Small pieces of yams were subjected to 45°C in a hot air oven for 50 mins. After the treatment the pieces of yams were kept at room temperature for 2 hours. Yams were dried in an oven at 100°C for 6 hours. They were ground to powder.

Isolation of free and bound form of diosgenin from powdered yam of *Dioscorea* :

The powdered sample of yam was refluxed with chloroform for 15-20 minutes and filtered. The filtrate contained free diosgenin. The chlorogorm part was concentrated to get free diosgenin.

The residue obtained after filtration of chloroform was hydrolysed with 11.3 (N) HCI for 3 hours. The slurry obtained offer hydrolysis was allowed to attain room temperature and filtered using vacuum in a Buchner funnel. The residue was frequently washed with distilled water till the filtrate was free from acid as indicated by the use of litmus paper. Thefiltered residue was transferred to a petridish and in an oven at 100^{0}C for 6 hours. It was extracted with petroleum ether (b.p. 40-60^{0}C). The extracted solvent was concentrated to a small volume, chilled in ice for sometime and filtered to obtain diosgenin that was present in bound form (Bammi and Randhawa, 1975).

Purification of diosgenin by TLC

Purification of diosgenin has been performed by thin layer chromatography taking silica genl G as adsorbent and chloroform acetone (3:1) as solvent mixture, the details of which has been represented in the chapter four.

Quantitative estimation of diosgenin

The quantitative estiation of free and bound form of diosgenin have been performed following the methodology involving colorimeter and reaction mixture of acetic acid resorcivol and conc. H_2SO_4. The details of the procedure has been represented in the chapter four.

Isolation of saponin from the yam of *D. prazeri*

500 gm of freshly cut yam of *D. prazeri* was crushed in an electrically operated mixture to form a paste that was mixed with 250 ml of water and boiled at 100°C for an hour under reflux condition. It was filtered to obtain water extract of the yam. The extract was concentrated and evaporated more or less to dryness. The solid mass left was treated with methanol and refluxed for 30 min. It was filtered and the methanol extract was concentrated to small volume, chloroform was added dropwise to prodce turbidity. It was kept overnight to obtain crystals in globular form. It was re-crystallised from methanol Chloroform mixture and the solid mass was collected as crude saponin.

Preparation of stock solution of saponin

Saponin was dissolved in distilled water after warming for a short lime till it was dissolved and 1000 ppm of saponin solution was produced.

Preparation of buffer solution :

Acetate buffer was prepared after adding 83 ml of 0.2 M acetic acid and was made upto 100 ml with 0.2 M sodium acetate solution to maintain pH at 4.0.

Extraction of enzyme from the yam

100 gm of high temperature treated yam, kept at room temperature for one hour was taken in a chilled mortar pastle containing chilled buffer solution (100 ml) and macerated thoroughly. The concentrated mass was then centrifused in cold and supernatent was decanted off, strained through four layers of cheese cloth and the homoginate was taken for further purification through dialysis.

Activity of enzyme of saponin to yield diosgenin

5 ml of saponin solution was taken in a number of conical flask and 2.5 ml of enzyme extract was added to each of the conical flask and kept in incubator at 35±1°C. Every ten minutes after, a flask was taken out and enzyme activity was stopped after direct heatng. It was concentrated under reduced pressure and spotted on the glass plate with a layer of silica gel. G. in the form of streak, developed in the solvent system of chloroform. Acetone (3:1) along with the authentic diosgenin. The developed chromatogram having diosgenin (Rf 0.54) and Saponin (Rf 0.00) was air dried. The zone of diosgenin, detected under UV light was scraped off and treated with glacial acetic acid, warmed for 2-3 minutes and centrifuged. The final volume was made upto 1 ml and diosgenin was estimated taking the reaction mixture of resorcinol,acetic acid and conc. H_2SO_4 following the procedure represented in the chapter four.

Table - 32: Production of free diosgenin in heat treated and untreated yam of *D. prazeri*.

Treatment	Total weight yam (g)	Yield of free diosgenin (g)	Percentage on dry weight basis
Heat treatment	100	1.20	1.20
Untreated	100	0.60	0.60

Results and Discussion

The table 32 shows the yield of diosgenin in heat treated and untreated yam of *D. prazeri*. The yield of free diosgenin in the yam untreated has been observed to become 0.60% on dry weight basis but increase of free diosgenin content (1.20%) has been observed in the yam when it was subjected to high temperature (45°C) for the period of 50 minutes.

Elujoba et al (1993) observed high yield of monohydroxy sapogenin in the seed of *Trigonella foenumgraecum* using a temperature of 45°C. Bhusari et al (1982) also observed an increase in diosgenin content during heat treatment of

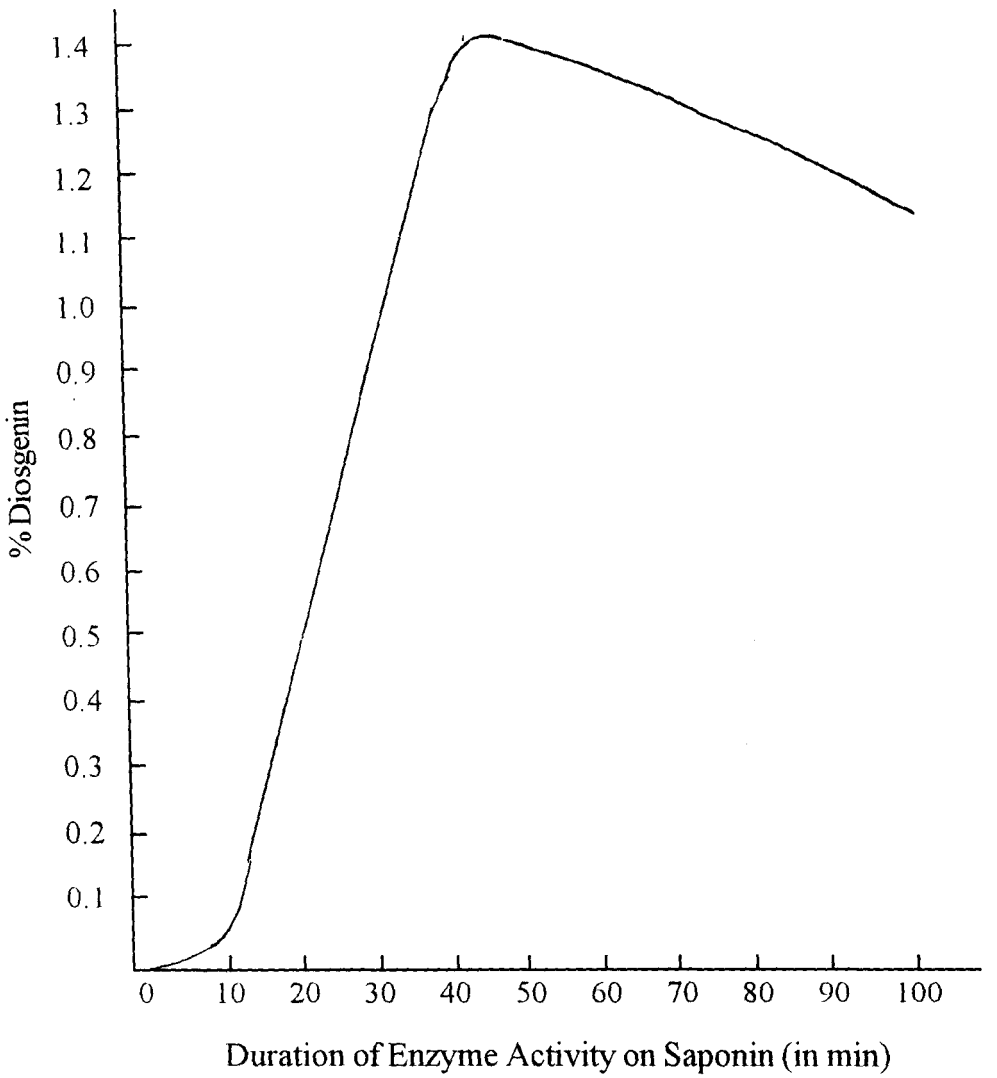

Fig. 45 : Acitivity of crude enzyme isolated from yam of *D. prazeri,* subjected to high temperature (45^{0}C) on saponin to yield diosgenin

seed of the plant. All these observations suggest the involvement of temperature effect on the activation of certain enzymes which may accelerate diosgenin production in the plant material. The result of the present investigation also supports the involvement of temperature in connection with the accumulation of diosgenin content. That the enzyme is a glycosidase and acts on saponin, is evident from the observation represented in the Fig.45. It has been observed that along with the increase of duration of enzyme activity, the free diosgenin has been observed to increase. Yield of free diosgenin becomes maximym of 1.40 percent on dry weight basis upto the limit of 50 minutes of enzyme activity after that the yield becomes low.

In the study on the chemical basis of temperature response in plants Ketellaper and Bonner (1961) observed cellular injury caused by unfavourable temperature and which might affect specific biochemical events related to diosgenin yield. Although this citations does not pin point any particular aspect of metabolism, it is equally possible that the activity of glycosidase becomes activated to supply the respiratory substrate obtained from glycoside during enzyme mediated hydrolysis. The released carbohydrate may in turn can be used as energy source by the plant material to cope with the adverse situation of accelerated aging of tusue caused by high temperature.

It has been pointed out earlier (Fig. 6, Chapter I) that during acid hydrolysis of saponin a considerable amount of diosgenin is lost due to its diene formation (Harborne, 1973). But no such loss of diosgenin occur during enzyme hydrolysis and which has been varified with the help of Thinlayer Chromatography. Thus there is a possibility of utilisation of enzyme hydrolysis for the production of diosgenin on industrial scale. This will result higher production. Besides it is expected that enzymatic process involve much less financial expenditure as compared to that involved during chemical process because the latter one always involve energy as well as huge chemical consumption.

Further research is very necessary to study enzyme kinetics and other problems related to the commercial production of diosgenin involving endogenous enzymes in the yam of *Dioscorea* sp.

SUMMARY

Small pieces of yams of *Dioscorea prazeri* has been subjected to high temperature of 45°C in a hot air oven for 50 minutes and kept at room temperature for 2 hours.

High temperature treated yam has been crushed to make powder from which free diosgenin has been extracted wtih chloroform.

Free diosgenin has been purified by TLC and estimated quantitavely by colorimetric method taking reaction mixture of acetic acid, resorcinol and conc. H_2SO_4 and measured at 510nm.

Heat treated yam showed higher amount of 1.20 percent as compared to that 0.60 percent in the untreated yam on dry weight basis.

It has been expected that the higher percent of free diosgenin in the yam treated wtih high temperature is due to enzyme hydrolysis of glycoside of diosgenin.

The glycoside of diosgenin has been identified to be dioscin after comparing mp. chromatogaphic behaviour and IR spectrum of isolated product with those of authentic sample of dioscin.

Enzyme has been extracted from yam treated with high temperature with the help of acetate buffer of acetic acid and sodium acetate maintained at pH 4.0.

Activity of crude enzyme on dioscin, the saponin during the period of 100 minutes to yield free diosgenin has been studied. Enzyme activity for 50 min has been observed to be optimum for yielding maximum percentage of free diosgenin.

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APPENDIX - I

AUTHORS PUBLICATION

1. PAPER

Investigation on Ethnic use of *Dioscorea* spp. available in Darjeeling and Sikkim Himalayas and Scientific evaluation of their traditional practice P. K. Basu and B. Gautam (2001).

In Proceedings of the National Seminar on Plant Biodiversity - Systematics, Conservation and Ethnobotany (in press)

2. ABSTRACT

Applicaiton of a newly established colorimetric method for quantitative estimation of diosgenin in some wild species of *Dioscorea* available in Kalimpong, West Bengal. P. K. Basu and B. Goutam

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INVESTIGATION ON ETHNIC USE OF *Dioscorea spp.* AVAILABLE IN DARJEELING AND SIKKIM HIMALAYAS AND SCIENTIFIC EVALUATION OF THEIR TRADITIONAL PRACTICE.

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Abstract

During survey on ethnobotanical practice in Darjeeling and Sikkim Himalayas, eight species of *Dioscorea* have been identified. The traditional use of all the species has been observed to involve the elimination of water soluble part of boiled yam used for edible purpose but utilisation of the same for the cure of wound infection in the skin of human body. Dioscin, a rhmnoglucoside of diosgenin has been isolated and identified as a toxic constituent in the water soluble part of the yam of *Dioscorea* the toxicity of the natural product is being claimed to be due to its lytic property as observed during anti-fungal activity against *Aspergillus niger*

INTRODUCTION

The knowledge of herbal medicine that has come through generations verbally is the main subject of ethnobotany. The term "Ethnobotany" was first used by Harshberger (1885) and its scope was much elaborated later (Ford. 1978; Faulks 1958) since then there has been a growing interest in the field (Jain 1986;Martin 1995) and has received much attention in certain parts of the world particularly in the underdeveloped and or developing countries where small or large portion of population still depend on natural resources in particularly indigenous condition and the impact of modern system of medicine has not reached them.

Botanically, the Darjeeling and Sikkim Himalayas may be said to be the richest source of medicinal and aromatic plants in India. Amongst the diverse floristic elements many plants are of religious, social and medicinal value. Their usage through the course of countless generations has rendered them to become indispensable part of culture of the people of the region, a sizeable population being comprised of the tribals.

Sir J.D. Hooker (1854) made the exhaustive floristic work on the Darjeeling and Sikkim Himalayas but he did not lay much stress on the healing properties of the indigenous medicinal plants. The most comprehensive work on medicinal plants in the region had to wait for many years (Biwas and Chopra 1940). Later observation on ethnobotanical studies in the region have been published (Bennet 1983; Tamang and Yonzone 1982; Yonzone *et al* 1984).But all the authors so far attempted in this line of work did mot mention *Dioscorea* as traditional or tribal medicines in the region. Though Nakamura (1998) did not mention the scientific name of "Turo" a traditional vegetable under Dioscoreaceae in Kyoto but according to him the plant had the ability to show bio-antimutagenicity.

From survey it appears that though modern system of medicine is available in the region, different species of *Dioscorea* are still being used by different tribal communities as ethnomedicine and various purposes.

This paper deals with discussion on present day use of different species of *Dioscorea* by the local as well as tribal people in the region.

MATERIALS AND METHODS

MATERIAL:

Eight different species of *Dioscorea* have been taken into consideration. These are: *D. alata* L., *D.kumaonensis* Kunth., *D.arachidna* Prain & Burkill, *D.sikkimensis* Prain & Burkill, *D.bulbifera* L., *D.esculenta* Burkill, *D.prazeri* Prain & Burkill and *D. sativum* L.

METHOD: FIELD WORK FOR COLLECTING OF PLANT MATERIALS AND INFORMATION.

Intensive field work has been done among different types of tribal populations i.e. Lepchas, Sherpas, Bhutias, Kami, Dorjee, Sarki, Sunar, Manger, Gurung, Ghate, Chettri, Bahun, Thakuri, Limbus and Rais available at fifteen different villages in Darjeeling district such as Tanek, Somalbung, Singi, Gorubathan, Mansong, Khari, Samthar, Nangsonden, Lolley, Gitdebling, Chhibo, Suruke, Sindebling, Bungbusty, Bijanbari, and seven villages in Sikkim i.e. Kerbari, Dentam, Ratomte, Singtam, Jorthang, Namchi, Rambhabung have been visited. Information on ethnomedicinal in the region has been collected from knowledgeable persons, medicine men and practitioner.

Some published and unpublished literature have been consulted for gathering information.

IDENTIFICATION OF PLANTS:

Plants and their parts were collected and herbarium sheets have been prepared. These have been identified after comparing with those of authentic specimens in the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal.

EXTRACTION, PURIFICATION AND IDENTIFICATION OF SAPONIN

Yam of *D. alata* was taken out from the soil and washed with water to free it from soil debris. The freshly cut yam was crushed in an electrically operator mixer to form a paste. The pasted yam was mixed with water and boiled at 100 °C for 1hr. under reflux condition. The water extract after filtration was evaporated to dryness. The solid mass was extracted with methanol and crystals were obtained from chloroform methanol following Harborne (1973). Various solvent mixtures such as Butanol:acetic acid : water (4:1:5), Methanol: Chloroform (9:1) and Phenol saturated with water were used for identification of steroidal constituents and sugars during paper chromatography.

PREPARATION OF POTATO DEXTROSE AGAR

Pure culture of P.D.A. was produced following Johnston and Berth (1983).

INOCCULATION OF CULTURE MEDIA WITH *Aspergillus niger*.

Two types of slants were used. In one type, *A. niger* was inoculated to freshly prepared P.D.A. in the test tube. In another tube the culture medium was previously mixed with 100ppm of the isolated dioscin in water and the slant was inoculated with the same fungus. The observation was noted after seven days, keeping both the types of slants at 25°C.

RESULTS AND DISCUSSION

It is established that majority of *Dioscorea* spp. are distributed in tropical and subtropical region all over the world including India. During survey eight different species of *Dioscorea* have been observed to be distributed mainly in the lower and middle hills in the region. It has also been observed that the region of Darjeeling and Sikkim Himalaya is occupied by the people belonging to different ethnic groups. It is apart from the three major ethnics –Bhutia, Lepcha and Nepalese, a conglomerate of over 20 ethnic tribes and a still more number of subtribes (Rai and Sharma 1996).

The climate in Northern part of Darjeeling and Sikkim Himalayas is dry and cold and most of the land is having higher altitude, which is beyond the range of monsoon resemble arctic region. In southern part of the region the climate is damp and warm at lower altitude such as is encountered in the tropical region. It is not surprising therefore that distribution of *Dioscorea* spp. and thick population of different tribal people are concentrated mostly in such tropical climate.

The Darjeeling and Sikkim Himalayas lie in that part of Asia where Nepal,China, India, Tibet and Bhutan are very close together. Though these areas have distinctive peoples. Climates, plants and animals but types appear in common and tend to intermingle occasionally where the boundaries of different great areas merge together. The region under study is roughly bounded by Nepal on the West flank, China-Tibet on the North and major part of the East. A part of the east is bounded by Bhutan and West Bengal is on the South.

From time immemorial the region was very much influenced by the Lepchas wonderful knowledge of the medicinal plants in the region (Biswas and Chopra 1940) the land form was inhabited by the Lepchas for a greater part of the history and Lepcha system of herbal medicine was predominant for a long time.

In Sikkim majority of the population 70% at present are from Nepali stock. The Bhutias and Lepchas constitute about 30% of the population. The original inhabitant in Darjeeling district was the Lepchas, which were rapidly outnumbered by settlers from Nepal and Sikkim. Once, the majority of the non-Indian nationals of Darjeeling was Tibetans (Economic Development Profile 1978).

From survey it appears that at present there are four different systems of traditional medicines available in the region – such as Lepchas, Nepalese, Tibetan and Bhutia system. Though Rai and Sharma (1996) mentionsd the availability of three systems of medicine but they did not mention the Bhutia system, though this system is being cultured at different places in the region.

Deeply seated in its antiquity but very thinly documented the Nepali system of herbal survives today in the region as "Jaributy" or simply as "Pahaday Dabai". The practise of berbal medicne has in its true nature not yet attained a system status in itself as in the case with Tibetan system, As because it is not yet organised set up. The "Jaributy" system has to bring up the several disjoined systems together to get proper status (Rai & Sharma 1996). From the survey it appears that Tibetan medical prescription usually comes in the forms of powder and different forms of extract much in contrast to Nepali medicine where the prescription normally is unprocessed plant product and comes in all of its natural shape, size, colour and form such as bark, twig, fruits, leaves seeds etc. representing traditional medicine. Though the Tibetan system had its root in ancient Tibet where spirituality dominated in practise but the general practitioner of Tibetan medicine at present relies more on the plants than spirituality.

TABLE-1: ETHNOBOTANICAL USE OF ***Dioscorea Sp.*** AS FOOD AND VEGETABLE IN DARJEELING AND SIKKIM HIMALAYAS

Plant species	Local name	Locality	Plant part	User	Preparation of edible part
D. alata L.	Khamalu	All locality	Rhizome & Bulbill. Rhizome	Local people including poor tribals. Poor people including tribals.	Rhizome / bulbill cleaned thoroughly boiled in water cooked or eaten as vegetable. Rhizome cleaned thoroughly boiled in water to make it a paste and used as alternative to rice.
D.sikkimensis Prain & Burkill	Niltarul	All locality	Rhizome & Bullbill	Local people including tribals.	Rhizome / bulbill cleaned thoroughly boiled in water and eat as vegetable.
D. bulbifera L.	Bantarul	All locolity	Rhizome & Bulbill	Tribals	Rhizome / Bulbill cleaned thoroughly boiled in water and eat as vegetable.
D.kumaonensis Kunth.		Turuk,Dentam, Samalbung, Samthar, Lolay, Gitdebling, Suruk,Namchi, Kerabari,Dentam, Singi, Singtam,Kham, Jorthang,	Young rhizome Rhizome & bulbill Old (mature) rhizome	Local people Poor local people Tribals	Young rhizome which lack fibres are used as vegetable. People eat both rhizome and bulbills after being boiled in water. Old rhizome, boiled in water are given to pigs as food.
D.esculenta Burkill.	Sutuni	Do-	Rhizome	All local people and tribals especially Lepchas.	Eat rhizomes after being boiled in water.
D. sativum L.	Githa	Lolay, Turuk, Gitdabling, Rambhabung, Jorthang.	Rhizome & Bulbill	All tribals especially Lepchas and Bhutias.	Though edible after boiling in water but local people do not eat regularly.

Table – 2: ETHNORELIGIOUS USE OF *Dioscorea* sp. By nepalese in darjeeling and Sikkim himalayas

Plant species	Local name	Locality	Plant part	User	Mode of use
D. alata L.	Khamalu	All localities	Rhizome	Nepali	All Nepali people eat a part of rhizome boiled to celebrate the festival of "Makar Sankranti" in the month of January each year.
D. sikkimensis Prain & Burkill	Niltarul	Do	Do	Do	Do
D. bulbifera L.	Bantarul	Do	Do	Do	Do
D. esculenta	Sutuni	Do	Do	Do	Do

Table – 3: ETHNOSPIRITUAL USE OF DIFFERENT SPECIES OF ***Dioscorea*** IN DARJEELING AND SIKKIM HIMALAYAS

Plant species	Local name	Locality	Plant part	User/Tribe	Purpose/mode of use
D. arachidna Prain & Burkill	Bharlang	Tanek, Chibo, Suruk, Samthar, Bungbusty,	Rhizome	Rais & Limbus	Fresh rhizome after being cleaned are eaten by the tribal people as per recommendation of Phedangmas (Limbu's doctor) and Bizuas (Rai's Doctor) commonly known as "Jhankri" the spiritualist in the region.

Table – 4: ETHNOMEDICINAL USE OF DIFFERENT SPECIES OF ***Dioscorea*** IN DARJEELING-SIKKIM HIMALAYAS

Plant species	Local name	Locality	Plant part	User/Tribe	Preparation	Purpose
D. sativum L.	Githa	Gorubathan, Mangsong, Tanak Bungbusty, Bijanbari, Surut, Samthar	Rhiome	All Tribal people	Rhiome boiled in water.	To kill worms present in stomach, to check gastric trouble
D. prajeri Prain & Burkill	Cucurtarul	Nangsondang,Tanak, Chibo, Khoni, Suruk, Gitdebling	Rhizome Rhizome	All tribal people Lepchas	Crushed rhizome boiled in water. Crushed rhizome boiled in water	Water solution of rhizome used all over the surrounding of wound for checking swelling and infection. Hair washing for killing lice, also used as fish poison.
		Turuk, Jorthang, Ratomale	Rhizome	Limbus, Rais, Tamangs, Chettris & Mangers.	Powder of dried rhizome	For abortion
D. bulbifera L.	Bantarul	All Localities.	Rhizome	Nepali people	Powder of dried rhizome	Piles, tumors, Asthma Bronchitis, Anthelmintic, abdominal pain.

Table –5: ETHNOVETENARY USE OF DIFFERENT SPECIES OF *Dioscorea* IN DARJEELING-SIKKIM HIMALAYAS.

Plant species	Local name	Locality	Plant part	User / tribe	Name of the disease and symptoms	Dosage/ preparation
D.kumaonensis Kunth		Sanaktsung, Singi,Lolay,Tanek, Khari,Suruk Samthar, Gitdebling	Rhizome	Lepchas, Sherpas, Bhutias, Kam, Chettri Dorjee, Sarki, Sunar Manger, Gurung, Bahun Ghate Thakuri	"Bhyagute' disease of cattle, the scientific name of which is Haemorrhagia septimae caused by "Pasteurella boviseptica" difficult in respiration producing some irritating sound, certain type of slippery fluid comes out from the noses, tears always comes out from the eyes, milk producing cattle stops to give milk, body temp. rise to 104°F-106°F, swelling of head, neck chest, excretory and reproductive organs, stool with blood, usually the animal die within 12 hours after infection.	Rhizome of the plant should be cut into pieces and be supplied into the mouth of the diseased cattle. This should be continued thrice daily for one week. Modern therapy sulphamezi-thin (5gm/day) or vaccination.
D.sativum L.	Githa	Tanek, Singi khari, Lolay, Gitdebling, Suruk	Rhizome	Lepchas, Bhutias, Sherpas, Mangers, Bahun, Sarkis.	Parasitic disease of cattle caused by *Fasciola hepatica.* Cattle becomes weak and eyes look yellow in colour, swelling of stomach.	Rhizome should be given to the infected cattle to eat.

Table-1 shows that six species of *Dioscorea* such as yams and bulbils *D. alata, D.kumanensis, D.sikkimensis, D. bulbifera, D.esculenta, D. sativum* ,are edible but *D.arachidna* and *D.prazeri* are nonedible types. It has also been observed that though *D. sativum* is considered edible but local people are not habituated with the regular use of the plant though the Lepchas and Bhutias use it regularly. Besides the mature yam of *D. kumaonensis* used by the tribal people is also given to pigs as feed after boiling in water. In connection with the *use of D.esculenta* it has been observed that though local people eat yams but only the Lepchas generally cultivate the species because the local people strongly believe that if the higher castes other than Lepchas cultivate it, generation of the cultivator may degenerate. It is also observed that Nepali people do not eat all the edible *Dioscorea* regularly but they eat specially the yam of *D.sikkimensis, D. bulbifera, D.esculenta, D. alata* to celebrate the festival of "Makar Sankranti" in the month of January in each year. **(Table-2)**

Tubers of certain species of Dioscorea are used as staple foods in a number of countries of West Africa, South America and the Indian subcontinent.

The edible species particularly, *D. alata* and *D. esculenta* are characterised by very high tuber yield. The chemical composition in the tuber of the edible types, particularly their nutritional values has been studied earlier (Coursey 1967). The starch constituted the major portion of the tuber. It was observed to become 10.59 % in *D. alata and 11.53% in D. esculenta.* Besides it has also been observed that *D. alata had 1.37%* of total sugar as compared to 4.08 % in *D. esculenta* . Besides, *D. esculenta* had more sucrose and glucose than those

of *D. alata*. According to Bammi and Randhawa (1975) total yields of sterol in the tuber of *D. alata* and *D.esculenta* are 3.37mg/100gmand 0.92mg/100gm respectively.

Worshipping for blessing and betterment of life is a practice common to all human beings. During worshipping traditional rituals are being performed. Since the time immemorial this customs are being observed in tribal communities in Darjeeling and Sikkim Himalayas. But there is no doubt that from ancient days only those plants are having with economic benefits they are being treated as sacred plants.

One of the primitive traits in the culture of the people of Darjeeling and Sikkim Himalayas is the existence of spiritualists known as "Jhankri" who also practice herbalism. In connection with ethnospiritual use of Dioscorea it has been observed that rhizome of *D. arachidna* is used by *Phedangmas* (Limbus' Doctor) and *Bizuas*(ais'Doctor) to kill evil spirit (**Table-3**). Common people believe that persons effected with ghost or evil spirit are having with less energy and vigour. It is expected that the yam of *D. arachidna* may contain some natural product having stimulatory property and which may be called mind altering substance.

From literature it appears that during fifteenth century A.D Europeans were habituated using a few mind altering substances. At that time the population had no coffee, tea, opium, cocane, tobacco etc. It has been reported by Lewis and Elvin-Lewis (1977), that substitute like roasted part of yam of Dioscorea was used as stimulating beverage. They also mentioned that tea made from the roots of *D. villosa* was taken by Meskwakis to relieve body pain of fatigue condition.

As regards the ethnobotanical use of different species of Dioscorea, the Lepchas, generally use the yam of *D.prazeri* for hair washing, to kill lice and fish poisoning,.But others like Limbus, Rais, Tamang, Chettris and Mongers use the water decoction of the yam of the species for curing wound of human being. They also use the powder of the yam to check population by induction of abortion. It has been observed that Nepali people use the yam of *D. bulbifera* for remedy against Piles, Bronchitis, Tumors, Asthma, and Dysentry. Besides, they also use it as anthelmintic and to relieve pain in the abdomen. (**Table-4**).

Min et al (1998) carried out ethnopharmacological investigation in Australia taking rabbit model to show that the root tuber of *D.opposita* had regulative effect on the thyroid disfunction of thytroidectomized animals.

Ethnovetenary use of different species of Dioscorea has been observed in the region (**Table– 5**). The yam *of D. kumaonensis* is commonly used as a remedy against "Bhyagute" disease of cattle by all tribal people specially the Lepchas, Bhutias and Sherpas in the region. This type of disease has a symptom of respiratory trouble of cattle producing irritation sound. Certain type of slimy fluid generally comes out as tears from eyes. Milk producing cattle generally stops to give milk. Body temperature rises to 104° –106° F. The surrounding areas of feet, neck, chest, excretory and reproductive organs swells up. Blood comes out with the stool. Generally animals dies within 12-14 hrs. after the initiation of the symptoms. For remedy against the disease yams of *D.kumaonensis* are cut into pieces and are forced to enter into the mouth of the cattle as feed. This is generally repeated thrice daily for one week. The scientific name of the Bhyagute disease is known as "Haemorrhagia Septimae". The name of the causal organism of the disease is *Pasteurella boviseptica*. In modern therapy Sulphamezitin (5gm) and vaccination is used. The rhizome of *D. satuvum* has been observed to become effective against parasitic disease caused by *Fasciola hepatica* in cattle and for which the animal becomes weak and their eyes look yellow with swelling of stomach. Yams of *D. sativum* are generally given to the diseased cattle to eat the yam.

Thus we find that most of the diseases which are caused by different microbes and parasites are cured by the use of yams of different Dioscorea and it is expected that all these species must have some natural product active against causal organism.

While working on isolation of steroidal constituents in the yam of different species of *Dioscorea*, it was observed that even in rainy season the water extract of the yam of *D. alata* did not show any contamination of micro- organism. Though the material extracted with other solvents kept in a container showed severe contamination with a fungal strain. The fungus was identified as *Aspergillus niger*.

In order to understand the nature of chemical inhibitor in the Yam of *D. alata* to serve as antifungal agent, chemical investigation was carried out following the conventional phytochemical method.

The crystals that were obtained after extraction of the yam of *D.alata* with water and subsequent methanol-chloroform treatment was observed to show melting point at 287°C. That the isolated product was a saponin,was confirmed by heavy frothing or soapy lather while boiling the natural product in water. The Rf values of true saponin i.e. 0.25and 0.45 in Butanol: acetic acid: water (4:1:5v/v/v upper phase) and Methanol: Chloroform (9:1 v/v) respectively have been observed to be the same as those of authentic dioscin. The IR spectrum of the saponin showed characteristic peaks (lambda) $_{max}$ 3350 (Broad) 1640, 1375, 1175, 1050, 850, 820, 720 cm^{-1} and were superimposed with those of authentic dioscin. The sapogenin was derived after following the procedure adopted by Harborne (1973). The sapogenin obtained after acid hydrolysis showed melting point 206-208 °C. The chromatographic behaviour and the observation on IR spectrum of the isolated sapogenin were observed to be the same as those of authentic diosgenin. After removal of diosgenin from the acid hydrolysed part of saponin, the filtrate was neutralised with Barium carbonate, concentrated and spotted on paper for chromatography. The Rfs of Rhamnose, 0.34 and 0.59 and those of glucose 0.13and 0.34 were observed to be the same as those of isolated products in the solvents of Butanol:Acetic acid : Water (4:1:5 v/v/v) and Phenol saturated with water respectively.

Fig.1A.shows that the slant without dioscin served medium for good growth of *A. niger* as reflected from the very good formation of white mat of mycellium. Whereas Fig.1B shows the dead mycellium of hyphae due to lysis of membrane. It is very interesting to note that the centrally placed dioscin free agar bearing inocculum shows white mat of hyphae: still in living condition.

The modern antibiotic era can be said to have opened on February 12, 1941 with the first clinical trial of Penicillin. This was followed by the introduction of one after another of major antibiotic substances, which remain the mainstay of clinical therapy of infectious disease to this date. Intensive screening has resulted in descriptions of more than 2000 individual antibiotic (Mitscher 1975).

Man has doubtless been aware at least dimly for centuries that anti-microbial agents are present in higher plants because the folk literature contains frequent references to such use, Stretching back for at least 6000 years. Large scale screening programme for anti-microbial agents from higher plants were undertaken in the 1940's using methodology which enables one to judge the relative worth of results. An examination of *Dioscorea bulbifera* from Western Samoa in the pacific area, Norton *et al* (1973) found that the plant to be of greatest interest especially in connection with its anti microbial activity.

While searching for biologically active plant ingredients by means of antimicrobial tests on oriental crude drug preparations, Imai et *al* (1967) observed that *D. toro* gave Gracillin and Dioscinprosapogenin as active agents. This is followed by the study of the activity of 23 steroidal aspogenin and saponins, which indicated that only saponins are active. According to them well known haemolytic activity of saponins probably prevent these agents from playing a role in systemic infections of humans.

Dioscin is a glycoside of diosgenin and is commonly present in the yam of *Dioscorea*. Structurally, it has chacotriose i.e. Rhamnose-Glucose- Rhamnose type of carbohydrate moiety linked with diosgenin. Though haemolytic property of Dioscin has not been worked out in details but the lytic property of Solamargine, which is, also Chacotriose of Solasodine – a nitrogen analogue of diosgenin, has been worked out in details. Solamargine cause significant disruption of phosphatidyl/Cholesterol, liposomes (Roddick *et al* 1990). There is a good evidence that membrane disruption by glycoalkaloids is a consequence of their binding to the free sterol components of membrane (Roddick 1987). Solamargine has been observed to show lytic effect on *Penicillium notatum*, derived from protoplast and bovine erythrocytes (Roddick *et al* 1990). Erythrocytes are much more susceptible to lysis. Solamargine caused 100% haemolysis at 20 micrometer. The significance of this phenomenon is generally related to Chacotriose nature of carbohydrate.

Recently, Puri *et al* (1993) confirmed the similar structural property of Solasodine and Diosgenin with the help of ^{1}H and ^{13}C assignment by two-dimensional NMR spectroscopy. According to them chemical shift of protons for both the chemicals are very close.

It is obvious, therefore, that haemolytic property of Dioscin may be attributed to the presence of Rhamnose-Glucose-Rhamnose combination in the carbohydrate and which is available in different Dioscorea (unpublished) showing ethnocedicinal value. Thus the traditional use of elimination of water-soluble part of yam of edible species and utilisation of yam with water-soluble part for the cure of wound infection and other type of infection may be claimed to be due to toxic constituent like Dioscin in the Yam of *Dioscorea*.

Recently, Beneytout *et al* (1995) claimed diosgenin as a new megakaryocytic differentiation inducer of HEL (Human Erythroleukemia) cells and observed that diosgenin addition to HEL TIB 180 Cell line induces morphological and biochemical changes characteristic for megakaryocytic cells assessed by cellular morphology, endomitotic process and glycoprotein content. Erythro-leukemia is a disorder that combines involvement of red cell precursor and myeloid precursor to show malignant alteration of several cell lines in the blood.

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গবেষণাপত্রের সংক্ষিপ্তসার
Abstract of Papers

পশ্চিমবঙ্গ রাজ্য বিজ্ঞান ও প্রযুক্তি সংসদ
বিজ্ঞান ও প্রযুক্তি এবং অপ্রচলিত শক্তির উৎস বিভাগ
পশ্চিমবঙ্গ সরকার,
উত্তরবঙ্গ বিশ্ববিদ্যালয়
এবং
পশ্চিমবঙ্গ বিজ্ঞান মঞ্চ

West Bengal State Council of Science & Technology
Department of Science & Technology and
Non-conventional Energy Sources
Government of West Bengal,
University of North Bengal
and
Paschimbanga Vigyan Mancha

পশ্চিমবঙ্গে অবস্থিত কালিমপঙ এলাকার বিভিন্ন বন্য ডাইয়োস্কোরিয়া প্রজাতির মধ্যে ডাইয়সজেনিন পরিমাপে এক নতুন ধরণের কলোরিমিটার পদ্ধতির ব্যবহার

বি. গৌতম এবং পি. কে. বসু
ফারমাকোগ্নসি রিসার্চ ল্যাবরেটরি,
উদ্ভিদ বিজ্ঞান বিভাগ,সেন্টার ফর লাইফ সাইন্সেস
উত্তরবঙ্গ বিশ্ববিদ্যালয়, শিলিগুড়ি - ৭৩৪ ৪৩০

বিভিন্ন ঔষধ প্রস্তুত করণে ডাইয়সজেনিন সাধারনত ১৬-ডাইহাইড্রো প্রেগনেনোলোন এ্যাসিটট তৈরী করতে বিশেষ কাজে লাগে। কারণ এর থেকেই তৈরী হয় জীবনদায়ী ঔষধ কর্টিসোন ও জন্মনিরোধক বড়ি। বর্ত্তমানে ভারতবর্ষে ডাইয়সজেনিন উৎপাদনের যে সমস্ত উৎস রয়েছে, ক্রমাগত লোক সংখ্যা বৃদ্ধির জন্য তা যথোপযুক্ত নয়। তাই বর্ত্তমানে ডাইয়সজেনিনের নতুন কোন উৎসসন্ধনানের প্রয়োজনের কথা চিন্তা করা হচ্ছে। দেখা গেছে ভেষজ উদ্ভিদের গুনাগুন বিচারের সময় অনেক সংখ্যক নমুনার বিশেষ পরীক্ষার প্রয়োজন হয়। তাই এই সমস্ত নমুনার মধ্যে ডাইয়সজেনিনের অস্তিত্ব এবং তার পরি মাপের জন্য চাই এমন এক সহজ পদ্ধতি যার ফলে খুবই কম পরিমান নমুনা দিয়ে এই অনুসন্ধান চালানো সম্ভব। তাছাড়া এই ধরণের পদ্ধতিতে অতি স্বল্প মূল্যের কলোরিমিটারে ব্যবহার করা উচিত যেটা যেকোন ল্যাবরেটরীতে সহজলভ্য হয়ে পড়ে।

এই গবেষনা পত্রে এক নতুন ধরনের এবং খুব কম সময়ে ডাইয়সজেনিন পরিমাপ করা যায় এমন এক পদ্ধতির কথা বলা হয়েছে। এই পদ্ধতির দ্বারা কালিমপঙের বিভিন্ন বন্য প্রজাতির ডাইয়স্করিয়ার মধ্যে ডাইয়সজেনিন পরিমাপের সম্ভাবনার কথা উল্লেখ করা হয়েছে।

APPLICATION OF A NEWLY ESTABLISHED COLORIMETRIC METHOD FOR QUANTITATIVE ESTIMATION OF DIOSGENIN IN SOME WILD SPECIES OF *Dioscorea* AVAILABLE IN KALIMPONG, WEST BENGAL

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In pharmaceutical industry, diosgenin is generally utilised for the preparation of 16-Dehydropregnenolone acetate which again is required for commercial production of cortisone and contraceptive pills. Now a days, the production of diosgenin out of conventional sources is not sufficient enough to cope with the rapid increase of population in India. For this reason searching of new source of it is very much needed. Besides, during evaluation of drugs it is required to handle a large number of samples and for which an easy and rapid method for estimation of diosgenin on microscale with the help of easily available low cost apparatus like colorimeter, is necessary.

This paper deals with the establishment of a new and rapid method for quantitative estimation of diosgenin, with the help of which wild species of *Dioscorea* available in Kalimpong has been investigated for understanding of their diosgenin content.