OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

FAIR USE POLICY

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

RESTRICTED

20238

DP/ID/SER.A/1653
8 June 1993
ORIGINAL: ENGLISH

135 p.
tables

PRODUCTION OF PHARMACEUTICAL MATERIALS FROM MEDICINAL AND AROMATIC PLANTS, PHASE II

DP/TUR/88/001/11-03

TURKEY

Technical report: Support to Anadolu University Medicinal Plants Research Centre*

Prepared for the Government of Turkey
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of B. N. Dhawan,
experimental pharmacologist

Backstopping Officer: T. De Silva
Chemical Industries Branch

United Nations Industrial Development Organization
Vienna

---

* Mention of firm names and commercial products does not imply the endorsement of the United Nations Industrial Development Organization (UNIDO). This document has not been edited.

V.93 86244

ABSTRACT

PROJECT TITLE: SUPPORT TO ANADOLU UNIVERSITY MEDICINAL PLANTSRESEARCH CENTRE

PROJECT NUMBER: DP/TUR/88/001/11-03

OBJECTIVE

1. To start biological evaluation of natural products and make functional, available equipment for this purpose at TBAM.

2. To train existing staff and develop protocols for biological evaluation in selected areas.

3. To organise and conduct a Workshop on Pharmacological Studies with Natural Products.

4. To make recommendations regarding future work of the Pharmacology Laboratory at TBAM.

5. To prepare a manual for use of experimental pharmacologists.

DURATION: Forty Four Days.

CONCLUSIONS AND RECOMMENDATIONS

1. TBAM has the necessary manpower and infrastructure for undertaking biological evaluation of natural products.

2. The future work should include broad based biological evaluation of pure constituents isolated at TBAM and of plants endemic to Turkey having drug potential. Secondly, detailed studies should be undertaken with plants having a demonstrated potential. At present, such leads have been obtained for hepatoprotective, spasmolytic and anti-urolithiatic activities. Appropriate protocols have been developed for fllow up.

3. The animal facilities need considerable improvement and strengthening. Necessary plans have been provided.

4. Short term training to two Pharmacologists and some additional equipment is necessary. These have been identified.

5. The Workshop was well attended and indicates both the keenness of Turkish Pharmacologists to work in this area and the linkages TBAM has built with other institutions and industry.

6. The manual prepared for the workshop can be adopted as a Field Manual after minor modifications.

C O N T E N T S

I. INTRODUCTION

A Centre for Research on Medicinal Plants (TBAM) has been functioning at Anadolu University, Eskisehir, Turkey since 1980. UNIDO support to its activities started in 1984, the first phase being completed in 1988 and phase II scheduled for completion in 1993. The Centre has been mainly involved in chemical and industrial research on cultivated medicinal and aromatic plants of Turkey. As a logical extension of these programmes, it was considered necessary to develop facilities for evaluation of biological activity of these natural products. The Centre has procured some equipment and has a part-time Associate Professor of the Faculty of Pharmacy and two full-time staff members available for initiating these studies.

I was requested by UNIDO in September 1992 to accept an assignment for 44 days, including travel time, briefing/ debriefing etc. at this Centre. The job description has been detailed in Annexure I.

I reached Eskisehir on 17th December, 1992, after a briefing (14th and 15th December, 1992) with Dr T. De Silva, Mrs. B. Pressinger and Mrs. De Roy at UNIDO Headquarters, Vienna and discussions with Mr. M. Toreli, Senior Programme Assistant in the Ankara Office of UNIDO (17th December, 1992). I left Eskisehir on 20th January, 1993, had detailed discussions with Mr. Toreli and Dr. M. Kamal Hussain, Director, UNIDO Office in Ankara the same afternoon. The debriefing at UNIDO, Vienna on 22nd January, 1993, involved a short meeting with Mrs. B. Pressinger and extensive discussions with Dr. De Silva regarding my activities at Eskisehir, the draft report and recommendations for follow up action.

The major objectives of TBAM is research and development work, including pilot plant studies, related to industrial processing and quality control of plants used as raw materials for pharmaceuticals, cosmetics, dyes, food

etc. It now plans to undertake a biological evaluation of the national flora and hence the needs for setting up a screening laboratory for necessary biological activities.

My original job requirement (Annexure I) included installation of procured equipment, training the local persons, conduction of a group training course and preparation of a manual for pharmacological screening. On reaching Eskisehir, it was seen that animal supply was a major limiting factor. Considerable time was devoted to improve the existing facility and to plan a dependable adequate sized animal house.

All the original objectives as well as subsequently added taskss have been successfully achieved.

II. THE MEDICINAL PLANTS RESEARCH CENTER (TBAM)

TBAM started as an Institute of Anadolu University on 5.10.1980 and became a Centre in 1982. The UNIDO support for developing pilot plant facilities for plant based drugs and pharmaceuticals was initiated in 1984. The new building was occupied in 1986 has 2000 sq.m. flocr area. The second phase of UNIDO support was initiated in 1988.

The Centre undertakes research and development work, including pilot plant studies, relating to the industrial processing and quality control of plant raw materials used in the preparations of pharmaceuticals, food, cosmetics, dyes, etc. Through its work in evaluating the Flora of Turkey, the Centre hopes to contribute to a rational exploitation of the plant resources of the country and to a development of the industries mentioned. The Centre also runs courses for people from these industries in pilot plant and quality control.

Through joint research projects, the Centre has been collaborating with Turkish and foreign scientists to study and test the constituents of plant based materials and has made joint publications.

Since 1988 TBAM has been organizing in-plant group training programmes titled "Training on the Utilization of Medicinal and Aromatic Plants in Pharmaceutical and Related Industries (TRUMAP)" under the auspices and joint sponsorship of the Government of Turkey and UNIDO. So far, in five programmes, 48 participants from 25 developing countries have been trained in 3-week comprehensive course which includes theoretical lectures, laboratory practicals, pilot plant experiments, field trips and round table discussions. The participants, with at least a B.Sc. degree in Pharmacy, Chemistry and Chemical Engineering and 2 years experience in the field of medicinal and aromatic plants, are admitted to the programme being selected from among those nominated by their respective governments. Expenses of the selected participants are fully met by the organization.

The Centre has also successfully implemented an UNIDO SIS project titled, "Assistance to the Laurel Oil Factory in Silifke, Icel" for the rehabilitation of a semi-installed, non-functional factory designed to produce essential oil from Laurel leaves which are abundantly available in the region.

The Centre has also been supported by the Government and University funds as well as other collaborative research programmes and its own commercial company. This company allows the Centre to generate income through services to the industry and for other commercial operations.

TBAM has staff of 25 Scientists (Annexure II) whose qualifications range from Pharmacy, Chemistry, Biology to Chemical Engineering. The Centre has the following Departments:

1. Phytochemistry Laboratory
2. Analytical Laboratory
3. Instrumental Laboratory
4. Multipurpose Pilot Plant
5. Pharmacology Laboratory
6. Library and Documentation/Information Section

Plant materials are first dealt with at the Phytochemistry Laboratory in order to establish process parameters which will eventually help designing the scale-up work.

Analytical laboratory is equipped with modern equipments such as GLC, HPLC, MPLC, TLC Scanner Chromatotron, UV, IR, GC/MS, Refractometer, Polarimeter, Autotitrator etc. A multinuclei-FT-NMR is expected soon. Hence chromatographic and spectroscopic methods are heavily used for process control, quality assessment and quality control of raw materials and finished products as well as research and development.

Pilot plant facilities include multipurpose pilot plant, pilot and semi-industrial scale equipment for grinding, extraction, distillation, steam distillation, fractional distillation, short-path distillation, chemical reaction, evaporation, vacuum evaporation, centrifugation, filtration, drying, spray drying, freeze drying. These facilities are used for scaling up of bench scale processes as well as technology adaptation and/or technology development work.

The newly established Pharmacology Laboratory has facilities for limited in vivo and in vitro biological testing. TBAM is planning to set up experiments primarily for the following activities: litholytic activity, hepatoprotective activity, spasmolytic activity and immuno stimulant activity.

A list of major instrumental facilities at TBAM is provided in Annexure III.

The Centre, being recognised as a focal point in the field of medicinal and aromatic plants, has recently been authorized by the Ministry of Health to conduct analysis on essential oils imported or exported.

An Information Unit with facilities for data processing and instant retrieval of information from international databanks has also been established. The Centre has on-line

linkages with DIALOG and STN databanks. It is also linked on-line with the mainframe computer of the University through internal modem. This is one of the national nodes of the EARN/BITNET network which links it with more than 3500 Universities and Research Institutions around the world. In August 1991, TBAM initiated the organization of a discussion list on medicinal and aromatic plants short-titled HERB in EARN/BITNET. Through BITNET access the Centre has also established on-line linkage with the NAPRALERT (Natural Products Alert) database of the University of Illinois at Chicago.

The Centre is creating two databases for the flora of Turkey. One is called FLOTURK. This database contains botanical, distributional, folkloric, chemical, analytical, pharmacological, procedural and trade information on each taxon. This database is estimated to be completed in five years time. The other database being created is DIGIL (Dedicated Information Generation for Institutional Libraries). This bibliographic database is based on published information on the medicinal and aromatic plants of Turkey. More than 1000 books, dissertations and reports in the library of TBAM and more than 3000 reprints of articles in the "Archives of Scientific Work done on Turkish Plants" are being abstracted in this database in English.

The Centre is national point of contact for the SCAMAP (South and Central Asian Medicinal and Aromatic Plants) network of UNESCO and should soon become a national node for APINMAP (Asian-Pacific Information Network on Medicinal and Aromatic Plants).

TBAM publishes "TAB Bulteni" (Bulletin of Medicinal and Aromatic Plants) since April 1989. The issue 7-8 has appeared in January 1993. The number of scientific publications has an increasing trend. In 1992, TBAM made 12 communications in 6 scientific meetings, published 24 papers in conference proceedings and 18 papers in International Journals. 26 more papers have been accepted for publication in International Journals.

The Director of the Centre is a member of the Turkish Pharmacopoeia Commission, UNIDO Consultant on Industrial Utilization of Medicinal and Aromatic Plants, member of the Editorial Board of Al-Biruniya and Khim.Prir.Soedin. (Chemistry of Natural Products) and member of the International Advisory Board of MAPA (Medicinal and Aromatic Plant Abstracts).

The services offeredtothe domestic industry include consultancy, troubleshooting, project design, process development, design engineering, feasibility studies, survey, assessment and rehabilitation of sick phytochemical units, quality assurance service, analytical control, research and development, production, information and documentation and training.

III. SUMMARY OF WORK DONE

A. Discussion with Staff Members

1. University Officials

I met Dr. Yilmaz Buyukersen, Rector of the University on 22nd December, 1992, accompanied by Prof. Baser. It was an useful meeting. He assured some immediate funds for procurement of cages for housing laboratory animals. He agreed, in principle, to consider construction of a new animal facility with modern amenities and wanted a plan to be developed for this building. The plan has been sent to Prof. Baser for further follow up action. A copy is enclosed (Annexure IV).

2. TBAM Scientists

I had initial detailed discussions with Prof. Baser and Dr. Yusuf Ozturk immediately on arrival on the 17th and 18th December, 1992 and subsequently at regular intervals regarding their programme of work and my own activities. The final discussions were held on 19th January, 1993. Prof. Baser also took me round the various facilities of the Centre including the Pilot Plant, Chemistry and Pharmacology Laboratories, Library and Animal Room.

Dr Ozturk told me about his research activities, his main interest of work being in the smooth muscle and also some other isolated tissues. He has recently studied the altered responses in smooth muscles from diabetic animals employing both streptozotocin and alloxan. He is assisted by Mr. Suleyman Ayden who has done M.Sc. in Pharmacology and had some traning in tissue culture and immunopharmacology at Munich, Germany. He would like to study some Turkish remedies for urolithiasis also. Another colleague Ms Rana Arslan is B.Sc. and will like to work for her M.Sc., but a specific problem has not yet been assigned to her. Dr. Ozturk has been to Regional Research Laboratory, Jammu for a few weeks and to Central Drug Research Institute, Lucknow for 3-4 days few years ago, but fell sick with infective hepatitis and thus could not fully utilize the opportunity. Moreover, RRL, Jammu was not a good choice for techniques to evaluate hepatoprotective activity.

Ms Berrin Bozan is a Chemical Engineer working for Ph.D. and is mainly interested in Arnebia densiflora which is rich in naphthoquinones. It is used locally as a coloring agent for food and wool. CDRI has done extensive work on a related species Arnebia nobilis. The arnebins isolated from this plant have anticancer, antimicrobial and wound healing properties, but some of them have a low LD50. The naphthoquinones isolated by Ms Bozan have been studied for anti-spasmodic activity and some are structurally different from CDRI arnebins. She has submitted the crude root extract and a pure compound to CDRI for further testing at my suggestion and I also advised her about further studies with the plant.

Mr. H.B. Sedat is the Chemical Engineer managing the pilot plant and is the seniormost full-time staff member. He too has been to India and discussions with him centred round his current activities. He is interested in plants having antidiabetic activity, particularly cloves. Brief discussions were also held with other staff members and their querries attended to.

3. Other Faculty Members of the University

I met several members of the Faculties of Pharmacy and Science during my stay. They wanted to discuss their present work and suggestions regarding follow up. Dr. Melinzey Tinoglu of the Faculty of Science has obtained Ph.D. from East Anglia University in U.K. and has been trained in muscle cell culture. His association with TBAM may be useful in utilisation of its tissue culture facilities. Dr. Nese Krimer of Faculty of Pharmasy has been working on Scotymus hispanicus whose 50% ethanol extract is extensively used in Turkey as an antispasmodic under the trade name Lityazol. UNIDO is supporting its standardisation and improved manufacturing facilities. It is also reputed to be anti-urolithiatic. Experimental procedures for its suitable evaluation were explained to her and her co-investigator Dr. Ipek Cingi. A protocol for evaluation of antiurolithiatic activity has been developed and left with Prof. Baser (Annexure IX). Ms Hulya Berberoglu is also working for her Ph.D. with Dr. Yozturk.

4. Medical School, Anadolu University

Even though it is located in a separate campus, the distance is manageable. I had several discussions with the Head of the Department of Pharmacology Dr. Ipek Cingi and Associate Professor Dr. Kevser Erol and visited their laboratory on 5th January, 1993. The faculty has two Associate Professors and 3 Assistant Professors. Their main interest is in diuretic, anti-inflammatory, spasmolytic and hepatoprotective activity of natural products. They utilise various smooth muscles (gall bladder, uterus, ureter, etc.) of sheep obtained from slaughter house in addition to rat and guinea pig tissues. Besides teaching medical students, they also teach students of nursing and run M.Sc. and Ph.D. courses. They have reasonable animal house facilities and do drug and hormone levels monitoring for the hospitalised patients with RIA procedures. They also collaborate with TBAM for pharmacological studies on some extracts and compounds isolated at TBAM. There is scope for substantially

increasing this collaboration. Dr. S. Alpan is interested in improving the animal maintenance and breeding facilities of the department and some suggestions have been given.

Associate Professor Dr. Nese Tuncel of the Department of Physiology has done very good work on haemorrhagic shock and the role of VIP and opioid peptides.

5. Department of Pharmacology, Faculty of Pharmacy, University of Arkasa

This was the only laboratory I could visit outside Eskishir due to lack of time and inclement weather throughout my stay. This is a well organised department with 11 faculty members and 7 postgraduate students. The Chairman Prof. V. Melib Altam was sick and Associate Professor Nuray Ari-Yildizoglu showed me the facilities. The department is mainly interested in responses of smooth muscles from diabetic animals to drugs particularly using in vitro aortic ring and intestinal preparations. A large amount of Ugo Basile equipment, like activity cage, rota rod, convulsometer, multichannel recorders, is available but perhaps not optimally utilised.

The animal house is maintained in a separate building and is better organised than elsewheree in the country. The cages and pellet food were also of a better quality. They breed the animals for their own use.

The department has a very good collection of routinely used drugs and provided many drugs as well as some animals for the workshop organised by me at Eskisehir.

I was particularly impressed by Ms Serap Gur who had just submitted her Ph.D. thesis, again on some responses of myocardium from streeptozotocin diabetic animals. The response of several agonnists including adenosine has been investigated. Animals with propylthiouracil induced hypothyroidism were also studied by her.

6. Pharmacologists from other Universities of Turkey

Prof. E. Sezik of the Faculty of Pharmacy, Gazi University, Ankara and his Associate Professor S. Yesilada have been working on anti-inflamatory and anti-ulcer activity of Turkish plants. The biological evaluation is undertaken by their collaborators in Japan. They were encouraged to set up simple tests in their own laboratory or to utilize the facilities of TBAM.

Dr. Mert Ulgan has obtained his Ph.D. in Pharmacokinetics from Kings College, London and is currently working at Marmara University in the Faculty of Pharmacy. He has worked extensively on amine metabolism and can be very useful in residue analysis of pesticides, diagnosis of poisoning etc.

B. Commissioning of Equipmments

Several equipments for the Pharmacology Laboratory had been procured but were not being used, some of them were still in their original packing. After reviewing the equipment, some were identified to be set up on a priority basis. The following equipments were set up and the staff members trained in their use:

1. Electroconvulsometer by Ugo Basile for screening of anticonvulsant drugs. The stimulation parameters for supramaximal electroshock seizure pattern test in mice were standardised.

2. Randall-Siletto Analgesiometer: Used for testing analgesic activity, particularly of antipyretic analgesics in rats, was made functional.

3. Ugo-Basile Plethysmometer: The assembly was set up and the procedure for measurement of paw volume in conscious rats standardised to be used for evaluation of anti-inflammatory activity of test materials.

4. Rota-rod for detecting motor deficit in mice treated with centrally acting drugs. Advice was given on modifying the Ugo-Basile apparatus, loaned from Ankara Facility of

Pharmacy, so that it could be fabricated in the workshop of the University at Eskisehir.

5. Eddy's Hot Plate Assembly, for assessing morphine like analgesic activity, was improvised with locally available materials and its working demonstrated to TBAM scientists. The design for an electric version was also provided.

Advice was also given regarding utilisation of several other equipments available in the laboratory and of some equipment planned to be procured.

C. Screening of Plant Products

There was no regular activity on-going in the laboratory. The Pharmacology Laboratory had not become functional after being transferred to its new location in TBAM building. After discussion with the staff members, it was deccided that the screening should be established in steps as the animal supply improves and other facilities become functional. In the meanwhile, certain tests could be started immediately with minor improvisations.

1. Acute toxicity: The protocol to be followed was standardised for testing to be done in mice. A proforma was developed and a beginning was made with known centrally acting agents.

2. Gross effects on CNS: Suitable cages were improvised, observation parameters and protocols were finalised and experiments performed with the following centrally acting agents:

1. Morphine
2. Pentobarbitone
3. Caffeine
4. Phenobarbitone
5. Chlorpromazine
6. Metrazol

Their doses were established for getting a demonstrable effect.

3. Special tests for analysis of CNS effects: All the test procedures were discussed in detail, the following procedures were actually demonstrated:

(a) In mice

i)	Motor coordination	- Rota Rod
		- Calalepsy
ii)	Hypnosis	- Righting reflex
iii)	Analgesia	- Haffner's test
		- Eddy's hot plate
iv)	Anticonvulsant	- Supramaximal electroshock seizure pattern test
		- Antimetrazol

(b) In rats

i)	Analgesia	- Randall Sileto
ii)	Anti-inflammatory	- Paw plethysmography

4. Use of isolated tissues: Dr Yusuf Ozturk was familiar with many procedures but their limited use was being made for screening procedures. The following preparations could be reset during my stay:

- Guinea pig ileum
- Rat duodenum
- Guinea pig auricle
- Rat uterus

Protocols were finalised for using these test systems in routine screening of natural products.

D. Development of Experimental Protocols

Detailed discussions with the staff of TBAM and a review of their on-going and proposed activities led to identification of the following areas of biological activities where testing could be initiated:

1. Acute toxicity and CNS effects
2. Cardiovascular effects
3. Hepatoprotective activity
4. Immunomodulator activity
5. Antiurolithiatic activity
6. Antispasmodics

In view of the long experience of Dr. Ozturk with the smooth muscles and his familiarity with experimental schedules, it was not necessary to develop a detailed protocol. A summary of the suggested line of work is given in Annexure V.

Detailed protocols have been developed for CNS effects, cardiovascular activity, hepatoprotectives and antiurolithiatic products (Annexures VI-IX). The experimental procedure for immunomodulator activity form a part of the protocol for hepatoprotective agents (Annexure VIII) and hence a separate protocol has not been appended.

I had carried with me a large amount of reference materials and I have left 62 reprints of my own work and other relevant publications to supplement these protocols and to provide necessary information in other related areas of screening for biological activity. A list is provided in Annexure X. I have also given a copy of a CDRI publication entitled, "Central Drug Research Institute R&D Highlights (1951-1990)" to TBAM.

Subsequently, I have sent copies of the following 3 books to Prof. Baser:

1. Bibliography of CDRI Research Contributions, 1951-90.
2. Compendium of Indian Medicinal Plants, Vol. I. R.P. Rasgoti & B.N. Mehrotra.
3. Compendium of Indian Medicinal Plants, Vol. II, R.P. Rastogi & B.N. Mehrotra.

E. Reorganisation of Animal Room

A small number of mice (about 100) and rats (about 10) are housed in 2 small rooms (approx. 2.5 x 3.5 metres) in poorly designed cages and under rather unhygienic conditions. It is necessary that the number of animals is substantially increased and their living conditions improved for satisfactory progress of the biological screening work.

Contacts were established with companies which were in a position to import polycarbonate or polypropylene cages for immediate needs. Catalogues and sample were obtained and an order has been placed for the first lot of these cages, with the help of funds Prof. Baser could procure from the University.

The immediate animal needs were assessed and based on these figures, the cage requirements were worked out. Details are given in Annexure XI. Some plastic cages were designed from locally available transparent lunch boxes to be used for gross observations and as maternity cages for mice.

It is necessary to have some guinea pigs and rabbits for in vitro screening tests. Limited supplies are available at the Department of Pharmacology of the Medical School and at the Faculty of Pharmacy, University of Ankara and a breeding nucleus could be procured from there.

The food pellets used are not satisfactory and lead to avoidable wastage. A better type of pellets were being manufactured at Ankara and Prof. Baser has been advised to switch over to these once the present supplies are consumed.

Discarded transfusion bottles were being used currently as water bottles. Most of these had abundant growth of some algae and were difficult to clean. Alternate smaller glass or plastic bottles have been recommended as replacement.

The present space is grossly inadequate for maintaining the required number of animals and will be a major constraint in biological screening. A laboratory vacated by Pharmacology (19 x 6.5 meters) adjacent to the animal rooms can be conveniently converted to be utilised for proper housing of laboratory animals. Prof. Baser had agreed to consider this and the necessary details have been left with Dr. Ozturk.

A note was developed on care of laboratory animals, including essential physiological norms, ethical considerations in animal experimentation and select bibliography. A copy is attached (Annexure XII).

The cleaning and maintenance of animal house is being presently done by the 2 associates of Dr. Ozturk. This takes a large proportion of their time and is not a satisfactory arrangement. Prof. Baser agreed to provide part time help as soon as possible.

F. Organisation of Workshop

A Workshop on Pharmacological Techniques for study of Natural Products was organised with the help of TBAM Centre staff on 12-15th January, 1993. The Workshop included 11 lectures and 4 demonstrations. A list of participants is given in Annexure XIII and the programme of the Workshop in Annexure XIV. The lectures were open to other members of the TBAM Centre and some of them did attend the lectures of their interest. In a special session on the last day, participants from outside TBAM made brief presentations about their current research activities and all of them exhibited interest in collaboration with TBAM. A manual (136 pages) was prepared for the participants in the workshop. A copy has been left with Dr. De Silva during the debriefing on January 22, 1993.

G. Deviations from Job Description

Considerable time had to be given for improving the animal facilities, finalising the cage procurement, planning conversion of old Pharmacology Laboratory in an animal facility and the layout of a new modern laboratory animal building.

IV. CONCLUSIONS

TBAM represents an excellent example of active multi-disciplinary group committed to optimal exploitation of local flora for medicinal and aromatic purposes. The emphasis so far has rightly been on technology development for value added products.

A major strategy in developing new drugs today is to search for molecular diversity. At the same time, the developing countries in particular, have to maximally utilise

locally available materials and technology to meet health care needs of their citizens at an affordable price. Natural products products provide valuable inputs for both these objectives in a cost effective manner. Turkey is endowed with varied and rich flora including a large number of endemic plants. Unfortunately, most of these await proper scientific evaluation of biological activity and further development as potential drugs.

TBAM provides a very good location for such an effort with necessary infrastructure for chemical isolation and characterization of active moities and for their large scale production. It has basic requirements for pharmacological studies in terms of materials and manpower. Marginal inputs for some pieces of equipment, training to two of their pharmacologists for a few weeks each and improvement in animal facility will lead to a good centre to meet the above mentioned objective.

Discussions with Biologists from other institutions in Turkey and a few enterpreneurs revealed that TBAM has built good linkages and enjoys good credibility. Most of the outside participants in the workshop were keen to collaborate with TBAM and this is an additional advantage in initiating biological evaluation of natural products at this centre. Several leads are already available with them and suitable protocols for biological evaluation have been developed during my stay at TBAM.

V. RECOMMENDATIONS

A. Programme of Work

The programme of biological evaluation should consist of two main lines of work. A general broad based biological screening containing a mix of suitable *in vitro* and *in vivo* test systems should be operated. To start with, it should include determination of acute toxicity, CVS and CNS effects and effects on selected smooth muscles. The Centre also has a few identified leads. These should be pursued for product development so that some positive outcome is quickly

obtained. For this purpose, these plants should be chemically studied in parallel with concerned bioassay to localise and characterize the active constituent(s). Subsequently, the pure constituents or a standardised extract can be developed as a drug.

1. General Biological Screening

TBAM has isolated essential oils from many plants (e.g. Calamintha nepata, Micromenia myrtifolia, Minuta florum, Origanum orites, Rosa damascena, Salvia fruticosa, Thymus subthorpii etc.) and most of these have not been biologically evaluated. Some of these are available in small quantities only. These should be screened first by in vitro testing on guinea pig ileum and tracheal chain preparations. The active essential oils can be prioritised for detailed study.

Turkey has around 1,000 endemic plants and most of these have not been evaluated for drug potential. TBAM should draw a priority list of about 100 plants based on availability (any threatened species may need priority), used in traditional medicine etc. These should be collected, botanically authenticated, extracted using standard procedures and 1-2 plants can be screened per month. The number could go up as animal facilities improve.

2. Detailed studies on selected plants

There are 4 plants, among those being currently studied at TBAM, which appear promosing and warrant a detailed study. I will suggest the following priority for these plants.

(i) Scolymus hispanicus: It is the main ingredient of a popular Turkish herbal remedy Lityazol used for treatment of renal and uretral calculi for 60 years. Preliminary in vitro studies at TBAM have shown its spasmolytic activity. This needs to be studied further using in vivo models and also on smooth muscles from other sites e.g. ureter, blood vessels, trachea etc. Its anti-urolithiatic activity should be demonnstrated using prootocols in Annexure IX.

(ii) Hypericum perforatum: The plant is used in Turkey for treatment of liver disorders and preliminary studies at TBAM suggest choleretic and hepatoprotective activity. These warrant a detailed study using protocols provided in Annexure VIII.

(iii) Arnebia densiflora: Published reports with other species, specially A. nobilis, indicate significant anti-microbial, anti-cancer and wound healing property in napthoquinones related to those found in A. densiflora. Preliminary studies at TBAM indicate spasmolytic activity in some of the locally isolated compounds. They should be investigated for other activities mentioned above.

(iv) Ecballium elaterium: The juice of this plant is used in Turkey for treatment of para-nasal sinus infection. Installation oof the juice in the nose leads to immediate copious thin watery secretion followed by a thick yellow, even blood tinged, secretion for several hours. The plant has been used in other countries as a hydrogogue cathartic. It is a well investigated plant chemically.

TBAM scientists have been advised to investigate the juice for effects on capillary permeability, ciliary activity, upper respiratory tract secretions and immune functions as well as possible antimicrobial activity. Some necessary references for experimental procedures have also been provided.

B. Procurement of New Equipment

TBAM has most of the equipment needed for biological evaluation available in the laboratory. A few critical equipments, however, needed to be added urgently. These are:

1. For CNS studies:
   - i) Actophootometer for spontaneous motor activity
   - ii) Rota rod assembly
   - iii) Electronic thermometer
   - iv) Electronic Y maze or reflex conditioner
2. Cardiovascular studies:
   - i) Electronic stimulator
   - ii) 4-Channel polygraph with suitable preamplifiers and transducers

iii) Blood pressure recording assembly for conscious animals

3. Hepatoprotective and antiurolithiatic activity:
   i) Clinical chemistry semiautomatic analyser
   ii) Flame photometer
4. Animal House:
   i) Additional cages and racks immediately
   ii) Animal weighing balances - 2
   iii) Incinerator and steam jet assembly for cage washing when new building is commissioned.
5. Proper Utilization of Existing Equipment

Most of the equipment available in pharmacology will be utilised once routine screening is initiated. The two major equipment which cannot be utilized for this work need redeployment.

1. Gamma Counter: The possibility of providing RIA facilities to neighbouring hospitals should be explored. Besides utilising the counter, it should bring some revenue as well to TBAM.

2. Cell Culture Assembly: Some basic studies may be initiated in collaboration with Dr. M. Tinoglu of Faculty of Science.

C. Training of Scientists

Dr. Y. Ozturk is a young bright and motivated Pharmacologist with an excellent track record. He should spend 4-6 weeks in a laboratory like the Central Drug Research Insttitute, Lucknow, India for training in organisation of biological screening programme and in specialised methods used for hepatoprotective and cardiovascular screens. His continued association with TBAM must, however, be assured.

Mr. s. Aydin is the only qualified Pharmacologist with full time attachment to TBAM. He needs training for anti-urolithiatic and CNS screens for 3-4 weeks and should also utilise the stay to familiarise himself with principles of laboratory animal breeding and husbandry.

D. Manpower Needs

The immediate requirement is for an Animal Attendant. A Technician may be needed in the Pharmacology Laboratory after a year or so when the scrreening gets fully established.

E. Development of Herbarium

TBAM is the most active Centre of research in medicinal and aromatic plants which is also developing a computerised database on Turkish plants. Its long term plans should include a modern herbarium of Turkish plants used as drugs or aromatics to serve as a centre of proper identification and authentication of investigated plant materials. Besides preserved herbarium specimens, colored photographs from actual growing locations should also be included.

Annexure I

JOB DESCRIPTION

The expert in collaboration with the National Project Director is expected to accomplish the following:

- Assess the current status of the in vitro and in vivo pharmacological testing facilities at MPRC.
- Assist in the installation and operation of the equipment already purchased.
- Train local counterpart staff in the use of in vivo and in vitro methods for bioactivity testing.
- Prepare in English a manual for pharmacological screening that could be used as documentation for a practical workshop.
- Conduct a group training course on biological screening techniques
- Assist in the establishment of standardized test methods and protocols for tests and reports.
- Recommend requirements for the improvement and development of the biotesting facilities at MPRC.

Finally, the expert will furnish a report embodying the progress made and outlining his recommendations to both UNIDO and the Government.

Annexure II

Post No.	Post title	Name and sex of incumbent	Date of joining
11-01	Director (Part time)	Mr K.H.C. Baser	11/83
Full Time			
11-02-1	Chem. Engineer	Mr S.H. Beis	12/85
11-02-2	Chem. Engineer	Mr T. Ozek	12/85
11-02-3	Chem. Engineer	Ms. Nezihe Azcan	11/91
11-03-2	Pharm. Analyst	Ms. M. Kurkcuoglu	5/85
11-03-3	Pharm. Analyst	Ms. Z. Erdemgil	4/90
11-03-4	Pharm. Analyst	Ms. M. Kosar	8/90
11-03-5	Pharm. Analyst	Ms. R. Arslan*	10/91
11-03-6	Pharm. Analyst	Ms. B. Demircakmak	9/92
11-04-1	Phytochemist	Ms. B. Bozan	12/85
11-04-2	Phytochemist	Ms. H. Tanriverdi	9/86
11-05-1	Plant Taxonomist	Mr. S. Aydin*	3/88
11-05-2	Plant Taxonomist	Ms. A. Kaya	7/88
11-06	Comp. Prog. Spec.	Ms. Berna Bozan	10/89
11-07	Documentalist	Not yet released	
11-08-1	Pharmacologist	Ms. N. Ermin	11/92
2	"	vacant	
3	"	"	
Part Time			
	Deputy Director	Mr M. Kara	2/84
	Pharmacologist	Mr. Y. Ozturk	1/87
	Analytical Chem.	Mr. M. Tuncel	1/87
	Pharm. Technol.	Mr. E. Guler	1/87
	Pharm. Technol.	Ms. Y. Yazan	1/87
	Pharmacognosist	Ms. N. Kirimer	11/86
	Pharmacognosist	Mr. M.Ogutveren	11/86
	Pharmacognosist	Ms. N. Ozturk	2/88
	Pharmacognosist	Mr. F. Demirci	10/92
	Pharm. Botanist	Ms. F. Koca	11/86
	Chem. Engineer	Mr. M. Kockar	11/86
	Chem. Engineer	Ms. E. Cakal	9/92

*Working in Pharmacology Section.

Annexure III

LIST OF EQUIPMENTS

a) Laboratory

1. Gas Chromatograph
2. Gas Chromatography. Mass Spectrometry System
3. High Pressure Liquid Chromatograph (3 pcs)
4. Medium Pressure Liquid Chromatograph
5. Infrared Spectrophotometer
6. Ultraviolet Spectrophotometer
7. TLC Scanner (densitometer)
8. Auto Spraying Unit for TLC plates
9. Chromatotron Centrifugal TLC System
10. Optical Electronic polarimeters
11. Refractometer
12. Auto Titrator
13. Freeze Dryer (2pcs)
14. Ultrasonic Bath (2pcs)
15. Moisture Balance
16. Analytical Balances
17. Muffle Furnace
18. Cryogenic Bath

b) Pilot Plant

1. 500 L S.S Multipurpose pilot plant (Extraction,evaporation and fractional distilation)
2. 100 L S.S. Fractional distillation pilot plant
3. 100 L Chemical reactor
4. 2000 L S.S Steam distillation plant
5. 80 L S.S Steam distillation plant
6. 60 L S.S. Soxhlet extractor
7. 3x500 L Battery of percolators
8. S.S Turbo drying oven
9. 1000 L S.S Steam jacketted Stirred vessel
10. Liquid. liquid extractor
11. Flash Evaporator
12. Sray dryer
13. Grinder
14. 5L Glass universal extractor
15. 200 L S.S vacuum evaporator
16. Large scale rotary evaporators (2pcs)
17. Steam generators (2 pcs). Total 1700 kg. h steam

c) Pharmacology

1. Deep Freeze Heraeus (German), upright ultra deep-freeze ($-90^{0}C$)
2. Sterilizer Heraeus. Air circulation sterilizer
3. Gamma-Counter Isodata 100 series with 5 well
4. Inverted microscope Olympus CK^{2} model
5. Microscope Olympus BHS model with BH2-RFC Reflected Light Fluorescence Attachment
6. Homogenizer Potter S homogenizer (Braun)
7. Centrifuge Heraeus Sepatch 17RS centrifuge. Up to 17.000rpm and 27000 xg is available. But due to lack of appropriate rotor type, it can be run max 25500 x g.
8. CO2-Incubator suitable [Heraeus]

9. Laminarflow cabinet [Holten]
10. Isolated Organ Bath Ugo-Basile (Italy). With two chambers.
11. Recorder Ugo Basile. Two Channel Recorder Gemini (for physiological recording)
12. Rodent Ventilator Ugo Basile (Italy)
13. Analgesy-Meter Ugo Basile (Italy) For Randall Selitto test
14. Plethysmometer Ugo Basile (Italy). For measurement of rat paw oedema
15. Bronchospasm Transducer Ugo-Basile (Italy).
16. Cardiotachograph Ugo Basile (Italy)
17. Strain-Gauge Preamplifier Ugo basile (Italy)
18. Pressure Transducer Preamplifier Ugo Basile (Italy)
19. Keller Pressure Transducer Ugo Basile (Italy).
20. ECT Unit Ugo Basile (Italy) for Small Mammals.
21. Transducers (Ugo Basile)
    - 24.1 Isometric transducer
    - 24.2 Isotonic transducer
    - 24.3 Cardiac transducer
22. Water Baths Thermomix UB (Braun) Bath and circulation thermostat and Frigomix S (Braun) Cooling bath with Thermomix BU on it.

d) Computers/Printers

1. IBM and IBM compatible (3 pcs)
2. Macintosh (4 pcs)
3. Laser Writer (2 pcs)
4. Dot Matrix printer (2 pcs)
5. Quietwriter

e) Camera and TV, Video sets

Transporter Land Rover 4 x 4

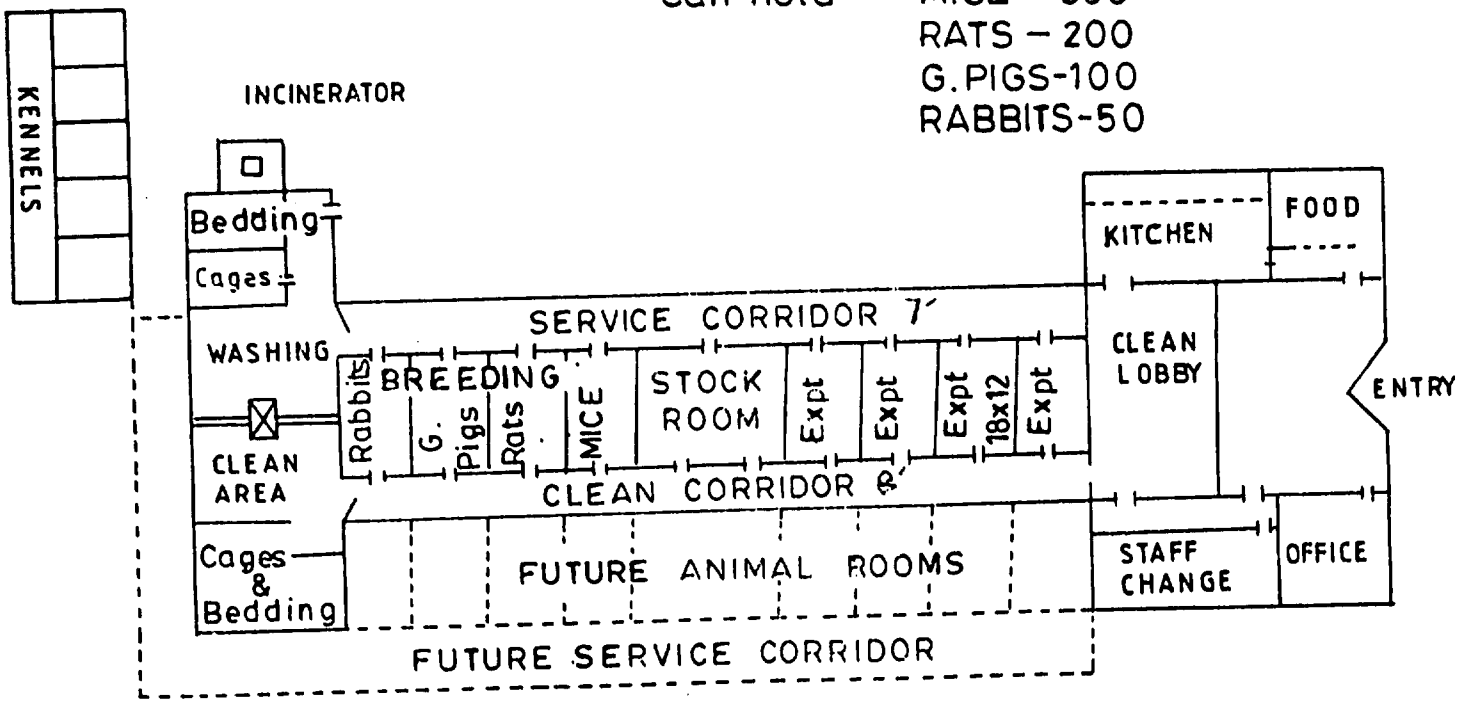
PLAN FOR ANIMAL HOUSE

ANADOLU UNIVERSITY
MEDICINAL PLANT RESEARCH CENTRE
ESKISEHIR, TURKEY

SCALE- 1INCH = 30 Ft. Approx.

[EACH ROOM FOR ANIMALS] Size Approx - 16 ft x 12 ft

Can hold — MICE — 300
RATS — 200
G.PIGS-100
RABBITS-50



Annexure V

PHARMACOLOGICAL STUDIES WITH ANTISPASMODICS

1. IN-VITRO ASSAYS ON GASTRO-INTESTINAL MUSCLES
    1. Spontaneous movements
    2. Chemically induced contractions
    3. Electrical stimulation

    Tissues from Rat, Guinea Pig, Rabbit, Man.

2. GASTRO-INTESTINAL MOTILITY
    A. Conscious Animals
        Rodents, Charcoal Meal
    B. Anaesthetized Animals
        Rabbit, Cat, Dog

3. EFFECT ON OTHER MUSCLES
    A. Smooth Muscle
        i. Blood vessel
        ii. Tracheobronchial
        iii. Uterus
        iv. Urinary Tract
        v. Biliary Tract

    B. Myocardium

    C. Skeletal Muscle

4. SYSTEMIC PHARMACOLOGY
        i. Acute Toxicity
        ii. CNS Effects
        iii. Local Anaesthetic
        iv. Diuretic

CDRI Publications on Pharmacology of Spasmolytic Compounds Isolated from Plants

BOENNINGHAUSENIA ALBIFLORA

1. S.H. Rizvi, A. Shoeb, R.S. Kapil and S.P.Popli
   Spasmolytic coumarins from *Boenninghausen albiflora*.
   Ind.J.Pharmaceut.Sci., 41, 205, 1979

CEDRUS DEODARA

2. K.Kar, V.N.Puri, G.K. Patnaik, R.N Sur, B.N. Dhawan, D.K. Kulshreshtha and R.P. Rastogi
   Spasmolytic constituents of *Cedrus deodara*, pharmacological evaluation of himachalol.
   J.Pharmaceut.Sci., 64, 258, 1975

3. V.N. Puri, K.Kar, G.K. Patnaik, B.N. Dhawan, D.K. Kulshreshtha and R.P. Rastogi
   Spasmolytic constituents of *Cedrus deodara* II. Isolation and evaluation of allohimachalol and three new sesquiterpenes.
   Ind.J.Exp.Biol., 13, 369, 1975

4. G.K. Patnaik, V.N. Puri, K.Kar, and B.N. Dhawan.
   Spasmolytic activity of sesquiterpenes from *Cedrus deodara*
   IDMA Bull., 18, 238, 1976

CLAUSENA PENTAPHYLLA

5. G.K. Patnaik, A. Shoeb, M.D. Manandhar, R.S. Kapil, S.P. Popli and B.N. Dhawan
   Spasmolytic activity of clausmarin, a new coumarin from *Clausena pentaphylla*
   Ind.J.Pharmacol., 10, 83, 1978

6. A. Shoeb, M.D. Manandhar, R.S. Kapil and S.P. Popli.
   Clausmarins A and B: Two novel spasmolytic terpenoid coumarins from *Clausena pentaphylla*
   Chem.Commun., 201, 1978

7. G.K. Patnaik, and B.N. Dhawan
   Evaluation of spasmolytic activity of Clausmarin-A. A novel coumarin from *Clausena pentaphylla* (Roxb.) D.C.
   J.Ethnopharmacol., 16, 127, 1982

COLEUS FORSKOHLII

8. M.P. Dubey, R.C. Srimal, G.K. Patnaik and B.N. Dhawan
Hypotensive and spasmolytic activities of coleonol, active principle of *Coleus forskohlii*
Ind.J.Pharmacol., 6, 15, 1974.

9. M.P. Dubey, R.C. Srimal, S. Nityanand and B.N. Dhawan
Pharmacological studies on coleonol, a hypotensive diterpene from of *Coleus forskohlii*
J.Ethnopharmacol., 3, 1, 1981.

CORYDALIS MELIFOLIA

10. G.K. Patnaik, D.S. Bhakuni, R. Chaturvedi and B.N. Dhawan
Cavidine, a spasmolytic agent from *Corydalis melifolia* Wall.
Indian Drugs., 21, 498, 1984

HERACLEUM THOMSONI

11. G.K. Patnaik, K.K. Banaudha, K.A. Khan, A. Shoeb and B.N.Dhawan
Spasmolytic activity of Angelicin: A coumarin from *Heracleum thomsoni (Linn.)*
Planta Med., 53, 517, 1987

MAXIMA ISOFLORA

12. M.S.R. Murthy, E.V. Rao, B.N.Dhawan and G.K. Patnaik
Antispasmolytic activity of certain maxima isoflavones
Ind.J.Pharmaceut.Sci., 48, 140, 1986

Annexure VI

EVALUATION OF ACUTE TOXICITY AND CNS EFFECTS OF NATURAL PRODUCTS

B. N. Dhawan

Central Drug Research Institute

Lucknow 226001, INDIA

With the revival of interest in natural products as an important source of new drugs it has become necessary to evolve suitable screening procedures. Such procedures should be capable of testing products of varying grades of purity and preferably utilise one species of animal. The data generated should be at least semi-quantitative and require small amounts of test material.

During the course of last 20 years CDRI has developed such a screening procedure for CNS effects largely based on tests in mice. Wherever any other species is used the doses can be extrapolated according to either of the following tables (Table 1 and 2) and approximately similar values are obtained with both the conversions.

Table 1. Extrapolation of doses between various species based on body weight.

		To				
		Mouse 20 g	Rat 150 g	Monkey 3 kg	Dog 8 kg	Man 60 kg
From	Mouse	1	1/2	1/4	1/6	1/12
	Rat	2	1	1/2	1/4	1/7
	Monkey	6	2	1	3/5	1/3
	Dog	6	4	5/3	1	1/2
	Man	12	7	3	2	1

Example: Mouse dose 120 mg/kg, Human dose 120 x 1/12 = 10 mg/kg

Table 2. Extrapolation of doses based on surface area to body weight ratio (K_m)

Species	Body Weight Kg	Surface area Sq m	Km Factor
Mouse	0.02	0.0066	3.0
Rat	0.15	0.025	5.9
Monkey	3.00	0.24	12.0
Dog	8.00	0.40	20.0
Man			
Child	20.00	0.80	25.0
Adult	60.00	1.60	37.0

Example: Adult human dose is 10 mg/kg = 10 x Km mg/Sq m = 370 mg/Sq m

Child dose = 370 / K_m factor = 14.8 mg/kg

Mouse dose = 370 / 3 = 123 mg/kg

For all the procedures described below adult mice (15-20 g) of either sex are used in groups of 5 animals each. The animal rooms as well as laboratories should be maintained at a constant temperature of 22-25°C. The test compounds are administered intraperitoneally dissolved or suspended with 1% gum acacia or 0.1% agar in distilled water. The initial testing is done with a crude (alcoholic or aqueous) extract. Active extracts are then fractionated and all fractions are tested for indicated activity. Then activity-linked separation procedure is followed till the compound(s) responsible for activity can be isolated and characterised. They are then taken up for a more detailed evaluation; if indicated.

ACUTE TOXICITY

In the initial stage an approximate LD_{50} (ALD_{50}) is determined using 2-3 animals per dose and 3 geometrically spaced doses (e.g. 46, 150 and 460 mg/kg) and recording the mortality in each group after 24 hours. The LD_{50} can then be estimated from the Tables prepared by Horn (H.J. Horn, Simplified LD_{50} (or ED_{50}) calculations, *Biometrics* 12, 312, 1956). More accurate LD_{50} determinations are only needed with products selected for detailed follow up study and will require the use of 10 animals

per dose and standard procedures (J.T. Litchfield and F.Wilcoxon, A simplified method of evaluating dose effect experiments, *J.Pharmacol.Exp.Ther.* 106, 319, 1949; D.J. Finney, **Probit Analysis**, Cambridge University Press, 1952)

EFFECTS ON CENTRAL NERVOUS SYSTEM

A. GROSS BEHAVIOUR: The materials are tested at 1/2 ALD_{50} dose for crude products and at 1/5 ALD_{50} for very pure fractions or single compound in all the CNS tests. The test material is administered to a group of 5 mice and a control group receives the vehicle only. The animals are observed for effects on behaviour every hour for 4-6 hours and then after 24 hours. The proforma used for this purpose is shown in Table 3. The test material can then be classified to fall in one of the following 3 groups on the basis of effects on GROSS BEHAVIOUR.

GROUP I: Stimulant Effect: The product may belong to either of the following categories

1. Antidepressant
2. Psychic stimulant
3. Convulsant
4. Hallucinogen

GROUP II: Depressant: Most of the plant materials show a depressant activity and these can be subclassified in 6 classes

1. Anxiolytic
2. Neuroleptic (Tranquilliser)
3. Narcotic analgesic
4. Centrally acting muscle relaxant
5. Hypnotic
6. General Anaesthetic

GROUP III: No gross effect. The material being tested may be INACTIVE. Before being declared inactive, however, the following types of activities have to be excluded.

1. Antipyretic analgesic
2. Antiepileptic
3. Antidepressant
4. Anorexigenic
5. Improving memory (Noo-tropic)

In some cases the gross effects are typical enough to characterise the type of CNS activity e.g. combination of catalepsy, hypothermia and diarrhea with reserpine; analgesia, miosis, respiratory depression and Straub's tail with morphine. In most cases it is necessary to perform additional confirmatory tests. For the sake of convenience these can be grouped in 3 categories. Only brief description is being provided below, since most of these are standard procedures. The battery of tests to be employed in a particular case will depend on its initial categorisation in one of the 3 groups described above.

B. DRUG INTERACTIONS

1. Antagonism of Amphetamine Effects: This group of tests helps in differentiating between anxiolytics, neuroleptics and hypnotics, all of which get classified as CNS depressants on the basis of their effects on gross behaviour. There are subtle differences in the gross behaviour and this will be described in a following section. Briefly, anxiolytics will not abolish righting reflex or corneal reflex but selectively block the pinna reflex, neuroleptics will produce hypothermia and catalepsy and hypnotics abolish righting reflex. Further characterisation is achieved by the results of the following 2 tests:

1.1. Amphetamine hyperactivity: Amphetamine is used in a small dose (5 mg/kg) and motor activity measured by an actophotometer or any suitable device. The hyperactivity is antagonised by all the 3 classes of CNS depressants mentioned above. The period of observation is 1-3 hours.

1.2. Amphetamine toxicity: This test requires 10 animals per dose and two different size of cages for housing the animals. The end point is percent mortality in 24 hours. In one group the animals are housed in a normal sized cage (non-aggregated animals) and in the second they are crowded together

in a small cage (aggregated animals). All animals are injected with 30 mg/kg amphetamine. Anxiolytic have no protective effect in these tests, neuroleptics selectively antagonise toxicity due to aggregation while hypnotics prevent mortality in both situations.

2. Effect on Barbiturate Hypnosis: Normally a short acting barbiturate like hexobarbitone (75 mg/kg) or pentobarbitone (45 mg/kg) is used and the duration of loss of righting reflex measured. The effect will be potentiated by most groups of CNS depressants and antagonised by analeptics and convulsants. If analeptic activity is suspected LD_{99} dose of pentobarbitone (130-140 mg/kg) is injected and the test drug given in $2xLD_{50}$ dose. Overnight survival of more than 40% animals suggests analeptic activity. Some plant extracts may indirectly alter the sleeping time by affecting hepatic metabolism of the barbiturates and some laboratories prefer to use barbitone (125 mg/kg) which is not metabolised by the liver.

3. Antireserpine Test: Animals are treated with reserpine (2.5 mg/kg) 3 1/2 hour before receiving the test product. Antagonism of ptosis, sedation, hypothermia (rectal temperature, preferably with an electronic thermometer) and diarrhea is monitored every half hour for the subsequent 3 hours and after 24 hours. Reserpine effect is specifically antagonised by depressant drugs.

4. Yohimbine Toxicity is selectively potentiated by antidepressants and this is considered confirmatory.

5. Antagonism of Chemical Induced Seizures: Seizures can be induced by strychnine (1.5 mg/kg) or metrazol (85 mg/kg) and they are antagonised by centrally acting muscle relaxants and antiepileptics (effective in petit mal) respectively. With small doses the time of onset may be prolonged with or without decreased intensity , with large doses mortality is also antagonised.

6. Drug-induced Writhing: Writhing can be induced by phenylquinone (10 mg/kg, E. Siegmund, R. Cadmus and G. Lu, A method for evaluating both non-narcotic and narcotic analgesics, *Proc.Soc.Exp.Biol.Med.*, 82, 328, 1953) or acetic acid (3%, 0.2 ml/mouse, R. Koster, M. Anderson and E.J. De Beer, Acetic acid for analgesic screening, *Fed. Proc.*, 18, 412, 1959). Some animals do not respond to these agents and it is necessary to pre-screen them 24 hours earlier, only the responders being included in the test. Some plant materials can themselves induce writhing when given by i.p. route, due to non-specific peritoneal irritation. This would become evident during gross behaviour study, in such

cases the plant extract should be given orally. Drug-induced writhing is abolished by antipyretic analgesics also, but they are inactive in most other tests for analgesia and thus get distinguished from narcotic analgesics.

C. SPECIAL TESTS IN MICE

1. Motor Activity: A qualitative assessment of the motor activity is only possible during the gross behaviour study and only major changes can be detected. A quantitative assessment is more reliable and can be obtained with actophotometer or any other device to diffrentiate between effect and no effect.

2. Forced Motor Activity: This is also known as the Rota-rod test (W.J. Kinnard and C.J.Kar, A preliminary procedure for the evaluation of central nervous system depressants, *J.Pharmacol.Exp.Ther.*, 121, 354, 1957). Mouse are trained to remain on a slowly moving (10 revolutions/min) rod (5-7 cm diameter) for 2 minutes. Animals treated with central muscle relaxants, hypnotics or general anaesthetics will fall down earlier.

3. Inclined Plane Test: It supplements information from the rota-rod test and measures ability of the animals not to fall from a flat wooden or glass plate inclined at 35° for 10 seconds (T.Kitano, K.Horikomi, J.Komiya, K.Yoshihara, H.Kawazura, S.Saito, K.Ozeki and A.M.I Zuchi, Studies on general pharmacological properties of sultopride, *Oyo Yakuri*, 26, 73, 1983).

4. Eddy's Hot Plate Test: The reaction time of mice placed on a hot (50 ± 0.5°C) plate is significantly increased by narcotic analgesics (N.B. Eddy and D.J. Leimbach, Synthetic analgesic II. Dithienylbutenyl and dithienylbutilamines., *J.Pharmacol.Exp.Ther.*, 107, 385, 1953). The animals must not be allowed to remain on the hot plate for more than 10-12 seconds to avoid burn injury in treated animals.

5. Supramaximal Electroshock Seizure Pattern Test: Clonic-tonic seizures are induced in mice by delivering supramaximal electrical current (48 ma, 0.2 sec) via ear electrodes. The tonic hind limb extension is abolished (E.A.Swinyard, W.C.Brown and L.S.Goodman, Comparative assays of antiepileptic drugs in mice and rats, *J.Pharmacol.Exp.Ther.*, 166, 319, 1952) by antiepileptic drugs useful in grand mal type of epilepsy (diphenylhydantoin, phenobarbitone). It should, therefore, be performed with extracts showing no gross effects or those producing depression.

6. Anorexigenic Activity: Fasted (24 hour) mice are given a measured amount of sweetened milk and the amount consumed in 15-30 minutes is determined. The consumption is reduced by anorexoginic agents and the test has to be done with extracts causing stimulation or having no gross effects.

7. Swimming Despair Test: Mouse left in a round water filled bath with no possibility of escape may stop swimming after some time, become immobile and even drown. This 'despair' is prevented by antidepressant agents (R.Porsolt, A.Bertin and M.Jolfre, Behavioral despair in mice: A primary screening test for antidepressants, *Arch.int.Pharmacodyn.*, 229, 327, 1977).

D. TEST IN RATS

The various groups of test procedures discussed above are adequate in characterising the activity of most of the extracts. Tests in rats are required for anti-inflammatory agents, drugs affecting memory and to distinguish between anxiolytics, neuroleptics and hypnotics to confirm the results of tests done in amphetamine treated rats.

1. Anti-inflammatory Tests: The most commonly used initial test is antagonism of carrageenan (0.1 ml, 1% solution, subplantar) induced hind paw edema (C.A. Winter, E.A. Risley and G.W. Nuss, Carrageenan induced edema in hind paw of rat as an assay for anti-inflammatory drugs, *Proc.Soc.Exp.Biol.Med.*, 111, 544, 1962) measured plethysmographically. The effective extracts need to be tested further in a subacute model like formalin (0.1 ml 2% solution, subplantar on days 0 and 2) arthritis (H. Selye, Further studies concerning the participation of the adrenal cortex in the pathogenesis of arthritis. *Brit.Med.J.*, 2,1129,1949) and ultimately a chronic model like the adjuvant (0.05 ml of 0.5% suspension of steam killed dried *Mycobacterium tuberculosis*) arthritis (B.B.Newbould, Chemotherapy of arthritis induced in rats by mycobacterial adjuvant., *Brit.J.Pharmacol.*, 21, 127, 1963). The response in these tests can be variable and it is necessary to include a group treated with a standard drug like phenylbutazone (50 mg/kg) or indomethacin (5 mg/kg). Further, the test materials should be administered orally since intraperitoneal administration may lead to endogenous adrenocortical discharge due to peritoneal irritation and thus give a false positive result. The use of other acute models like arachidonic acid induced ear edema (J.M.Young, D.A.Spires, C.J.Bedord, B.Wagner, S.J.Bollaron and L.M.De Young, The mouse ear inflammatory response to topical arachidonic acid, *J.Invest.Dermatol.*, 82, 367, 1984) is much more limited and not very suitable for routine screening studies.

2. Ulcerogenic Index: All non-steroidal anti-inflammatory agents produce varying degree of gastric irritation and it is necessary to assess it before selecting an extract with anti-inflammatory activity for a detailed follow-up.The extract is adminstered orally to fasted animals in antiinflammatory dose for a few days, animals sacrificed and severity and incidence of ulcers recorded (J.Thuillier, P.Bessin, F.Geffroy and J.J.Godfroid, Chimie et pharmacologie de la clofezone, *Chim. Therap.*, 3, 53, 1968) to calculate the ulcerogenic index (R.C.Srimal and B.N. Dhawan, Pharmacology of diferuloylmethane (curcumin), a new nonsteroidal anti-inflammatory agent. *J.Pharm.Pharmacol.*, 25, 447, 1973). The study may be conveniently combined with the subacute model like the formalin-induced arthritis.

3. Randall-Siletto Test: This is a further confirmatory test for analgesic activity, usually of antipyretic drugs. It measures the pressure over inflammed (0.1 ml of 2% yeast suspension or 1% carrageenan solution) hind paw necessary to produce pain and increase in threshold by analgesic or antiinflammatory drugs. The pressure normally required to elicit a response is 150 mm Hg in normal paw and 60-70 mm Hg in inflamed paw (L.O.Randall and J.J.Siletto, A method for measurement of analgesic activity on inflamed tissue., *Arch.int.Pharmacodyn.*, 111, 409,1957).

4. Yeast Induced Pyrexia: Pyrexia induced by injection of 2 ml Brewer's yeast suspension (15%) or TAB vaccine has been utilised to measure antipyretic activity of aspirin like compounds. It requires careful standardisation and should only be undertaken with products selected for preclinical development. The pyrexia with yeast is stabilised after 24 hours.

5. Conditioned Avoidance Responses: Rats can be trained to develop conditioned or secondary conditioned avoidance responses (G.Maffi, The secondary conditioned response of rats and the effects of some psychopharmacological agents, *J.Pharm.Pharmacol.*, 11, 129, 1959). The secondary response is specifically blocked by anxiolytics and conditioned response by neuroleptics while hypnotics block conditioned as well as unconditioned responses. Recently we have measured the period for training animals and shown that its reduction is a reliable and easy method for identifying plant products improving memory and learning (H.K.Singh and B.N.Dhawan, Effect of *Bacopa monniera* Linn. (Brahmi) extract on avoidance response in rat., *J.Ethnopharmacol.*, 5, 205, 1982; H.K. Singh, R.P. Rastogi, R.C. Srimal and B.N. Dhawan, Effect of Bacosides A and B on avoidance responses in rats, *Phytother. Res.*, 2, 70, 1988). Conversely, the period may be prolonged by agents like hyoscine (0.5 mg/kg ip), ethanol (40%, 10 ml/kg oral) or electroshock (80 mA, 100 Hz, 0.4 ms width, 200 ms, ear electrodes) which produce amnesia. During the past few years similar models have been developed in the mice as

well (K.Nishizawa, H.Saito and N.Nishiyama, Effects of Komakhi-To, a traditional Chinese medicine on learning and memory performance in mice, *Phytother.Res.*, 5, 97, 1991) but more experience is necessary before including them in a screening protocol. Numerous other procedures have been used and details will be available in books dealing with screening methods.

6. Y-Maze: This is used as a final confirmatory test for extracts affecting learning and memory (Singh and Dhawan, cited above). Briefly, animals have to choose between a darkened (natural preference, but get shock) and a lighted (to avoid shock) alley, the time for learning and number of wrong choices being monitored. The process is facilitated by noo-tropic agents.

E. TEST IN CAT

Mono- and polysynaptic reflexes like the patellar reflex and crossed extensor reflex respectivaly are recorded in anaesthetised cats. The polysynaptic reflexes are selectively blocked by central muscle relaxants (B.N.Dhawan, R.C.Srimal and J.N.Sharma, Selective inhibition by glycine of some somatic reflexes in cat, *Brit.J.Pharmacol.*, 44, 404, 1972). Besides needing sophisticated stimulating and recording equipment, the procedures requires considerable experience and should not be used routinely.

ORGANISATION OF SCREENING AND CHARACTERISATION OF CENTRAL EFFECTS

It is necessary for any laboratory initiating this activity to first familiarise itself with the effect of known drugs belonging to various groups of centrally acting agents. A summary of the positive responses is given in Appendix 1. It clearly indicates that each group has a characteristic spectrum of activity. Examples from natural products have been given wherever possible. Once this programme is established and standardised, plant extracts can be screened and adequately classified for follow up or dropped from further consideration. It should not be necessary to subject any extract to more than 3-4 tests, generally all in mice, and 100-200 mg extract should be adequate. The procedure has proved very useful at our institute and over 4000 plant materials and 500 marine flora and fauna extracts have been tested and a number of promising leads obtained. The protocol also includes several parameters like salivation, piloerection, lachrymation etc. which are more affected by cholinergic or adrenergic drugs. These have not been included in the present discussion but they add to the utility of the protocol as a part of a broad based screening programme.

Table 3: PROFORMA FOR RECORDING EFFECTS ON MOUSE BEHAVIOUR

Date Extract No/Name Concentration mg/ml
Room Temperature.... ^{o}C Dose mg/kg
Route i.p./oral
No of animals ------

Parameter	Time of observation after drug administration (hours)						
	0						24
A. GROSS EFFECTS 1. Motor Activity 2. Respiration 3. Ataxia 4. Tremor 5. Convulsions - Clonic - Tonic							
B. POSTURE & TONE 1. Body 2. Limbs 3. Tail (Straub) 4. Catalepsy							
C. EYE 1. Pupil (size) 2. Ptosis 3. Lachrymation							
D. REFLEXES 1. Pinna 2. Corneal 3. Righting							
E. RECTAL TEMPERATURE ^{o}C							
F. OTHER EFFECTS 1. Reactivity to - Sound - Touch 2. Piloerection 3. Cyanosis 4. Salivation 5. Diarrhea 6. Analgesia							

Parameter	Time of observation after drug administration (hours)						
	0						24
G. SPECIAL EFFECTS 1. Stereotypy 2. Agression 3. ..... 4.							
H. Mortality (number dead)							

Score as - 0, +, + +, + + +, no change, mild, moderate, strong
- v decreased, ^ increased
- A absent, P present

Animal No	Weight (g)	Volume of drug injected (ml)	EFFECTS
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

CNS EFFECTS - Stimulant/Depressant/No effect

SPECIAL TESTS NEEDED
1
2
3
4
5

Date

Signature of Investigator

APPENDIX 1

ANTIPYRETIC ANALGESICS

GROSS BEHAVIOR

No effect

DRUG INTERACTION

Antagonise phenylquinone/acetic acid writhing

OTHER TESTS

No effect

TESTS in RAT

1. Effective in all anti-inflammatory tests
2. Ulcerogenic
3. Antagonise yeast pyrexia

EXAMPLES

None from plants

Aspirin

Curcumin * from *Curcuma longa* is anti-inflammatory but not antipyretic

---

*1. R.C. Srimal and B.N. Dhawan, Pharmacology of diferuloyl methane (curcumin), a new non-steroidal anti-inflammatory agent, *J. Pharm. Pharmacol*, 25, 447, 1973

2. R. Srivastave, V. Puri, R.C. Srimal and B.N. Dhawan, Effect of curcumin on platelet aggregation and prostacyclin synthesis, *Arzneim. Forsch.*, 36, 715, 1986

3. B.N. Dhawan, M. Pande, R.C. Srimal and R. Srivastava, Antithrombotic effects of curcumin, *Thrombos. Res.*, 40, 413, 1985

NARCOTIC ANALGESICS

GROSS BEHAVIOR

1. Motor activity and respiration depressed
2. Straub's Tail positive
3. Pupil constricted
4. Analgesia

DRUG INTERACTION

Phenylquinone writhing blocked

OTHER TESTS

Reaction time in Eddys Hot Plate and Randall Siletto Test increased

EXAMPLES

Morphine

Codeine

GENERAL ANAESTHETICS

GROSS BEHAVIOR

1. Markedly depress or abolish motor activity and reactivity to sound and touch
2. Flaccid paralysis of body and limbs
3. Respiratory depression, cyanosis with large doses
4. All reflexes abolished
5. Analgesia

DRUG INTERACTIONS

1. Potentiate barbiturate hypnosis
2. Antagonise all convulsants

SPECIAL TESTS

1. Not necessary and not possible in mice and rats
2. In cats block both mono- and polysynaptic reflexes

EXAMPLES

None from plants

Thiopentone

CENTRAL MUSCLE RELAXANTS

GROSS BEHAVIOR

1. Decreased motor activity
2. Ataxia, body and limbs flaccid
3. Pinna and corneal reflex blocked, righting reflex blocked only with high doses

DRUG INTERACTION

Antagonise strychnine convulsions

SPECIAL TESTS

1. Increased fall out in rota rod and inclined plane test
2. Block polysynaptic reflexes in cat

EXAMPLES

None from plant sources

Mephenesin

ANTIDEPRESSANTS

GROSS BEHAVIOR

1. Spontaneous motor activity

   increased or no effect

2. Rectal temperature

   may be increased

3. Piloerection ±

DRUG INTERACTIONS

1. Reserpine effects antagonised
2. Yohimbine toxicity potentiated

SPECIAL TESTS

Swimming despair time prolonged

EXAMPLES

No natural product available

Imipramine

PSYCHIC STIMULANTS

GROSS BEHAVIOUR

1. Increased motor activity
2. Respiration stimulated
3. Tremors ±
4. Pupil dilated (amphetamines) or normal (caffeine)
5. Rectal temperature raised ±
6. Piloerection
7. Stereotyped behaviour, aggression may be present

DRUG INTERACTIONS

Antagonise barbiturate hypnosis

EXAMPLES

Caffeine

Ephedrine

CONVULSANTS

GROSS BEHAVIOUR

1. Increased motor activity
2. Respiration stimulated
3. Cyanosis
4. Convulsions

- clonic - strychnine
- tonic - metrazol

DRUG INTERACTIONS

Antagonism of convulsions by - Mephenesin
- Diazepam
- Barbiturates

	Mephenesin	Diazepam	Phenobarbitone
Clonic	+	+	+
Tonic	-	±	+

SPECIAL TESTS

None

EXAMPLES

Picrotoxin

Strychnine

HYPNOTICS

GROSS BEHAVIOUR

1. Depress motor activity
   respiration
2. Ataxia
3. All reflexes depressed or abolished
4. Reactivity to sound and touch depressed
5. Cyanosis with large doses
6. Body relaxed, limbs flaccid
7. Rectal temperature may be lowered

DRUG INTERACTIONS

1. Potentiate barbiturate hypnosis
2. Antagonise convulsants

SPECIAL TESTS

Increased fall out in rota rod and inclined plane test

EXAMPLES

Valerian

ANXIOLYTICS

GROSS BEHAVIOUR

1. Motor activity decreased
2. Ataxia
3. Body relaxed
4. Pinna reflex abolished, corneal reflex present

DRUG INTERACTIONS

1. Antagonise amphetamine hyperactivity
2. Potentiate barbiturate hypnosis
3. Antagonise strychnine convulsions in large doses

SPECIAL TESTS

1. Increased fall out in rota rod and inclined plane test
2. Block secondary conditioned response in rat
3. Polysynaptic reflexes blocked in cat

EXAMPLES

None from plant sources

Diazepam

NEUROLEPTICS

GROSS BEHAVIOUR

1. Motor activity decreased
2. Catalepsy
3. Ptosis
4. Righting reflex not abolished
5. Hypothermia
6. Reactivity to sound and touch not altered
7. Diarrhea with reserpine

DRUG INTERACTIONS

1. Antagonise amphetamine hyperactivity and toxicity in aggregated mice
2. Potentiate barbiturate hypnosis

SPECIAL TEST

Block secondary conditioned and conditioned response in trained rat

EXAMPLES

Reserpine

ANOREXIGENICS

GROSS BEHAVIOUR

No effect except with amphetamines (see under psychic stimulants)

DRUG INTERACTIONS

Nil

SPECIAL TESTS

Marked decrease in milk intake of fasted animals

EXAMPLES

None from natural products

Fenfluramine

Amphetamines (also produce CNS stimulation)

ANTIEPILEPTICS

GROSS BEHAVIOUR

No effect except with large doses of phenobarbitone (See under hypnotics)

DRUG INTERACTION

Metrazol seizures antagonised by drugs useful in Petit Mal epilepsy (Trimethadione, Phenobarbitone)

SPECIAL TEST

Extensor phase of supramaximal seizure abolished by drugs effective in Grand Mal epilepsy (Diphenylhydantoin, Phenobarbitone)

EXAMPLES

No natural product used clinically

Diphenylhydantoin (Grand Mal)

Trimethadione (Petit Mal)

Phenobarbitone (both types of epilepsy)

DRUGS AFFECTING LEARNING and MEMORY

GROSS BEHAVIOUR

No effect

DRUG INTERACTIONS

Nil

SPECIAL TESTS

1. Conditioned avoidance response

Training period significantly reduced by noo-tropic (improving memory) and increased by amnesic (impairing memory) agents

2. Y-Maze

Performance improved by noo-tropic agents

EXAMPLES

Hyoscine (Amnesic)

Bacosides [**] (Noo-tropic) from *Bacopa monniera* Lim.

A synthetic noo-tropic Piracetam is available for clinical use

---

**1. H.K. Singh and B.N. Dhawan, Effect of *Bacopa monniera* Linn. (Brahmi) extract on avoidance response in rat, *J.Ethnopharmacol* 5, 205, 1982

2. H.K. Singh, R.P. Rastogi, R.C. Srimal and B.N. Dhawan, Effect of Bacosides A and B on avoidance responses in rats, *Phytotherapy Res.* 2, 70, 1988

Annexure VII

SCREENING OF NATURAL PRODUCTS FOR CARDIOVASCULAR ACTIVITY

B.N. Dhawan
Central Drug Research Institute
Lucknow 226001, INDIA

Cardiovascular diseases are among the major killer diseases and also lead to a lot of morbidity. There is a continuous effort, therefore, globally to develop better drugs for their management. A large number of new drugs for cardiovascular diseases has been discovered from natural products and screening of plant materials for cardiovascular effects continues to attract worldwide attention. Both *in vitro* and *in vivo* procedures have been developed and can be combined into an effective cardiovascular screen. The main types of cardiovascular activities holding promise include blood pressure reduction, cardiotonic, antiarrhythmic, hypolipidaemic and antithrombotic actions. The procedures used for assessment of these activities will be briefly discussed.

A-BLOOD PRESSURE LOWERING

These agents have potential utility in the management of hypertension and mention may be made of Rauwolfia alkaloids and coleonol (M.P.Dubey, R.C.Srimal, S.Nityanand and B.N.Dhawan, Pharmacological studies on coleonol, A hypotensive diterpine from *Coleus forskohlii*, *J.Ethnopharmacol.* 3, 1, 1981), which is also known as forskolin, from plant sources. The experimental schedule may be conveniently divided in four parts.

1. Primary Screening in Anaesthetised Animals: These are acute experiments and also help in detection of many autonomic effects. The animal species commonly used are cat, dog and rat. The animals can be anaesthetised with pentobarbitone (35-40 mg/kg iv for cat and dog or ip for rat). Cat and dog may also be anaesthetised with chloralose (70-80 mg/kg iv). The blood pressure is usually recorded from a cannulated common carotid artery connected to a mercury manometer (via a tube containing 5-10% sodium citrate solution) or a pressure transducer (normal saline containing 100 U/ml heparin) for recording on a kymograph or polygraph respectively. The respiration, from cannulated trachea, is similarly recorded via a Marey's tambour (kymograph) or transducer (polygraph). The heart rate can be recorded on an ECG machine or polygraph. In the case of the cat, contraction of nictitating membrane in response to electrical stimulation of preganglionic sympathetic nerve (10 Hz, 1 msec, 5-10 V for 5-10 sec) is recorded via a frontal writing lever (kymograph) or a transducer (polygraph). After getting stable base line records, responses to intravenously administered (via cannulated femoral or

jugular vein) adrenaline (2-4 μg), acetylcholine, histamine and isoprenaline (1-2 μg each) are obtained. The plant extract is injected intravenously in a dose of 25 mg/kg and the responses to various pressor and depressor agents and, in the case of cat, contraction of nictitating membrane, obtained again. Depending on the response, a higher (maximum 50 mg/kg) or lower dose is tested. If there is marked effect, intraduodenal administration is also done. A repeat administration should be performed on recovery to exclude tachyphylaxis. In general, only a sustained lowering of blood pressure without marked effect on autonomic responses indicates the need of further follow up. If ECG is being recorded, arrhythmias may often be observed with extracts containing cardiotonic glycosides associated with transient hypertension or irregular changes in the blood pressure.

2. Studies in Conscious Normotensive Animals: Active products should be studied in conscious animal. Depending upon the facilities and the quantity of the material available studies can be done in trained dog or monkey where an ordinary blood pressure apparatus with a paediatric cuff can be used. Alternatively rat can be used and blood pressure measured by tail plethysmography. The effect of oral and repeated dose administration should be measured in these experiments.

3. Studies in Hypertensive Animals: Genetically hypertensive (Y. Yamori, Physiopathology of the various strains of spontaneously hypertensive rats. In:Genest et al Eds. **Hypertension**, McGraw Hill, Book Company, New York, p.556, 1983) rats are the best animals for these studies but the maintenance of such a colony needs special care and experience. Rats with neurogenic (P. Zandberg, Animal models in experimental hypertension: Relevance to drug testing and discovery, In: P.A. van Zwieten Ed. **Handbook of Hypertension**, Vol.3, **Pharmacology of Antihypertensive Drugs**, Elsevier Science Publishers, Amsterdam, p.6, 1984), nutritional (A.C.Guyton, The relationship of cardiac output and arterial pressure control, *Circulation*, 64, 1079, 1981) or renal hypertension are alternative choices. The experimental regimen is similar to that used for normotensive animals. An effect in hypertensive animals provides a better indication of clinical antihypertensive activity. Further, the effect is generally more marked in hypertensive than in normotensive animals.

4. Studies to Determine the Site and Mechanism of Action: The experiments in anaesthetised animals do provide some indication about the site and mechanism of action of blood pressure lowering agents, particularly if they are affecting the autonomic mechanisms. Reduction in response to adrenaline, for example, indicates α-adrenergic blockade, β-blockers reverse the adrenaline response and reduce that of isoprenaline while a ganglion blocking agent will block the nictitating membrane contraction, the blood pressure responses to various agents remaining unchanged or getting potentiated. A direct relaxant of smooth muscle of blood vessels will reduce the response to all the agents. The effect can be confirmed in an aortic strip preparation from the rabbit (R.F. Furchgott, Spiral cut strip of rabbit aorta for *in vitro* studies of responses of arterial smooth muscle, *Meth.Med.Res.*, 8, 177, 1961), perfused rat hind limb or with an electromagnetic flow meter in an α-adrenergic blocked cat (M.P.Dubey et al, 1981, cited above).

Many antihypertensive agents lower the blood pressure by acting an central vasomotor locii. The site can be localised by ablation (decerebrate, spinal or pithed animals), localised injection (vertebral artery for medulla, lateral cerebral ventricle for hypothalamus and medulla, intrathecal for spinal cord or microinjection in vasoactive nuclei), topical application (ventral medulla, floor of 4th ventricle) or altered responses to electrical stimulation of vasomotor loci (B.N. Dhawan, M.B. Johri, G.B. Singh, R.C. Srimal and D. Viswesaram, Effect of clonidine on the excitability of vasomotor loci in cat, *Brit.J.Pharmacol.*, 45, 17,1975; R.C. Srimal, K. Gulati and B.N. Dhawan, On the mechanism of central hypotensive action of clonidine, *Canad.J.Physiol.Pharmacol.*, 55, 1007, 1977). It is also necessary to study effects on cardiac haemodynamics (cardiac output, dP/dT, left ventricular pressure, electrocardiogram etc., R.C. Srimal, D.F.J. Mason and B.N. Dhawan, Pharmacological studies on a new hypotensive agent 3-γ(-p-fluorobenzylopropyl)-2,3,4,4a; 5,6-hexahydro-1(H)-pyrazino (1,2-a) quinoline hydrochloride (compound 69/103, Centpyraquin), Part II. Effect on cardiac dynamics., *Arzneim. Forsch.*, 28, 1092, 1978) and regional blood flow. The later can be measured with electromagnetic flow meter (M.P.Dubey et al, cited above), or more accurately and in small areas like the brain nuclei by using radioactive microspheres (A.Gulati, R.C.Srimal and B.N. Dhawan, Alterations in systemic hemodynamics and regional blood flow by isoprenaline, *Ind.J.Med.Res.*, 88, 262,1988).

B-CARDIOTONIC ACTIVITY

Cardiotonic drugs are one of the very few classes of therapeutic agents where plant products hold almost a total monopoly. Digoxin is the most commonly used glycoside but several other glycosides are available having a longer duration of action or a quicker onset and therefore available for emergency management by parenteral route. Any new cardiotonic agent will have to be compared with digoxin before gaining therapeutic acceptance. A judicious combination of *in-vitro* and *in-vivo* procedures is employed for this purpose.

1. In-vitro Preparations: The positive inotropic activity can be demostrated in isolated perfused heart (rat, guinea pig or rabbit depending upon the availability, O.Langendorff, *Pflugers.Arch.ges Physiol*, 190, 280, 1895), isolated guinea pig or rabbit auricle (A.J.Lock, Observation on the use of isolated auricle for the determination of inotropic potency, Brit.J.Pharmacol., 21, 393, 1963) or the isolated papillary muscle from the cat or guinea pig (M.Catell and H.J. Gold, The influence of digitalis glycosides on the force of contraction of mammalian cardiac muscle, *J.Pharmacol.Exp.Ther.*, 62, 116,1938). The cardiotonic glycosides also inhibit the Na^+K^+ATPase (T.Akera and T.M. Brody, The role of Na^+, K^+ -ATPase in the inotropic action of digitalis, *Pharmacol.Rev.*, 29, 187, 1977) and this can be quantitatively estimated in enzyme preparation from the heart or the brain.

2. In-vivo Demonstration of Cardiotonic Activity: The crude preparations found active in the *in-vitro* test are pursued further. They can be injected intravenously in anaesthetised cat or dog (described in the section on hypotensive agents) and the positive inotropic effect recorded by open chest myocardiography (N.N.De, M.M. Vohra and J.D.Kohli, A comparative pharmacological study of new cardiac glycosides from *Thevetia nerifolia*., In:W.Wilbrant Ed. Proced.First. Internat.Pharmacol.Meeting, Pergamon Press, Oxford, 1963, p.227). The animal must be put on artificial respiration before opening the chest. An older, but still practiced, method consists in determining the minimal lethal dose (Hatcher dose) in anaesthetised animal by giving intravenous injection at a rate producing cardiac arrest in 40-60 minutes in guinea pig or cat (G.K. Patnaik and B.N. Dhawan, Pharmacological investigations of asclepin, a new cardenolide from *Asclepias curassavica* I. Cardiotonic activity and acute toxicity, *Arzneim.Forsch.*, 28, 1095, 1978). The Hatcher dose provides quantitative data for comparison with standard glycosides.

3. Effect on Hypodynamic Myocardium: The *in-vitro* and *in-vivo* procedures outlined above (except the Hatcher dose determination) can be utilised. The isolated perfused heart can be made hypodynamic by low calcium perfusion, the papillary muscle by continuous maximal electrical stimulation and anaesthetised animals by intravenous injection of a large dose of pentobarbitone or phenylbutazone (Patnaik and Dhawan, cited above). All these preparations simulate the clinical failing heart and provide additional supporting data for further processing of the extract to isolate and characterise the active constituent. The studies described below are only indicated if the pure compound has possibility of being developed as a drug based on its yield, economics, comparative data in tests performed so far etc.

4. Effect on Cardiac Hemodynamics: These experiments can be satisfactorily performed only in larger animals like cat and dog. Various indices of cardiac activity like the cardiac output, left ventricular pressure, end-diastolic pressure, dP/dT, pressure velocity curve are computed by measuring the force of contraction, systolic and diastolic blood pressure, heart rate, electrocardiogram etc. in anaesthetised open chest animals on artificial respiration using suitable transducers and a multichannel recorder. If necessary the animal can be made hypodynamic. Comparative data should also be generated with a standard glycoside like digoxin (G.K.Patnaik and E.Kohler, Pharmacological investigations of asclepin, a new cardenolide from *Asclepias curassavica.* II.Comparative studies on the inotropic and toxic effects of asclepin, g-strophanthin, digoxin and digitoxin, *Arzneim. Forsch.* 28, 1368, 1978). These are difficult experiments and considerable experience is needed before dependable results can be obtained.

5. Determination of Safety Margin: This is another important parameter and should be determined both *in vitro* and *in vivo*. Cardiac glycosides are characterised by a narrow margin between the effective and the toxic dose and a new glycoside may not be clinically acceptable unless a distinct advantage can be demonstrated over available glycosides. The safety margin is determined by slow infusion at a constant rate, either in isolated atrial preparation or in an anaesthetised animal, and determining the relationship

of minimum effective, maximum effective and arrhythmic concentration/dose to the lethal concentration/dose. Asclepin, a glycoside isolated and evaluated at CDRI, for example, has much higher safety margin than many therapeutically used glycosides (Patnaik and Kohler, 1978, cited above).

6. Determination of Absorption, Cumulation Characteristics: These studies should be performed in guinea-pigs or cats only in the case of candidate glycoside to be developed as therapeutic agent, since a large number of animals is required. The *gastrointestinal absorption* is determined by giving the intravenous Hatcher dose orally, and observing the animals for 2-3 hours for mortality. If all the animals die, the oral absorption is 100% as has been reported with O-acetyl-solanoside (M.M.Vohra, G.K.Patnaik, R.S.Kapil and N.Anand, Chemistry and pharmacology of a glycoside of *Vallaris solanacea*, *J.Pharm.Sci.*, 55, 1425, 1966). If animals do not die, i.v. Hatcher dose is determined and the reduction in the dose will indicate the amount absorbed from the gastro-intestinal tract. The persistance of action is monitored by giving HD_{50} and then determining the Hatcher dose on each succeeding day. The reduction in Hatcher dose represents the amount remaining in the body. An alternative approach consists in giving HD_{20} dose daily and measuring the survival period. The animals should survive indefinitely with glycosides having no cumulation and die on the 5th day if there is total cumulation. Most glycosides give a value in between these two extremes (Patnaik and Dhawan, 1978, cited above).

C-ANTIARRHYTHMIC ACTIVITY

Quinidine was the first clinically used antiarrhythmic agent. This was also a natural product but subsequent attempts to get other antiarrhythmic agents from plant sources have not yielded fruitful results. A number of cardiac depressants has been isolated but they did not fulfil the required criteria of efficacy and safety. Secondly, several new synthetic compounds have been developed for the management of arrhythmias.

Cardiac arrhythmias can be of auricular or ventricular origin, hence any screening schedule should cover both these varieties. The procedure should include a mix of *in vitro* and *in vivo* techniques, inducing arrhythmias by electrical stimulation, chemical agents or by injury. A comprehensive review of the procedures has been published some time ago (L.Szekers and J.Gy Papp, **Experimental Cardiac Arrhythmias and Antiarrhythmic Drugs**, Kiado Academy, Budapest, 1971). A brief summary of important and commonly employed procedures has only been provided.

1. Isolated Guinea-pig Auricle: The preparation is set up in oxygenated Ringer-Locke solution at 37°C. The auricles are stimulated by rectangular pulses of 0.5 msec duration and 5 times the threshold (1.5-3 V). The frequency starting at 0.2 pulses/sec is increased at the same rate every ten seconds till the auricles fail to follow each impulse (G.S.Dawes, Synthetic substitutes for quinidine,

Brit.J.Pharmacol.Chemother., 1, 90, 1946). A Grass type of electronic stimulator is necessary for this experiment. The maximal rate of stimulation is determined before and after the addition of the extract, which should remain in the bath for about ten minutes. Quinidine is used as the standard drug.

2. Isolated Perfused Heart: The Langendorff preparation from a guinea-pig or rabbit can be used, arrhythmias being induced by cardiac glycosides like ouabain (B.R.Lucchesi and H.F.Hardman, The influence of DCI and related compounds upon ouabain and acetylstrophanthidine-induced cardiac arrhythmias, *J.Pharmacol.Exp.Ther.*, 132, 372, 1961).

3. Chemically-induced Arrhythmias in Anaesthetised Animals: These experiments can be performed in rat, guinea-pig, rabbit or cat. Briefly, arrhythmic drug infused slowly intravenously at a constant rate and ECG is monitored. While only the onset of arrhythmia and cardiac arrest can be monitored in the rat and guinea-pig, onset of early arrhythmias, ventricular tachycardia, ventricular fibrillation and cardiac arrest can be monitored in the cat. One group of animals is treated with the test drug, second group with an equal volume of normal saline and the third with quinidine (5mg/kg). The increase in the dose of arrhythmic agent to produce the various ECG changes indicates the activity of the test drug. The arrhythmias can be induced with ouabain (4 μg/min, guinea-pig), aconitine (4 μg/min, rat) or 10% solution of calcium chloride or barium chloride (rabbit and rat). In the cat 20 μg/ml solution of ouabain or aconitine can be used, the rate being decided by the ECG changes (K.C.Mukherjee, K.Kar, R.N.Sur and B.N.Dhawan, Antiarrhythmic property of beta-N-di-n-propylaminoethyl-O-methoxyphenyl ether hydrochloride (EUM), *Ind.J.Exp.Biol.*, 8, 22, 1970).

4. Electrically-induced Arrhythmias: Anaesthetised open chest cats on artificial respiration are used. The myocardium is stimulated with a Grass stimulator using two needle electrodes placed in the left ventricle 1cm apart, giving square wave pulses of 2 msec, 20 Hz for 10 sec. The starting voltage is 1 V and it is progressively increased till fibrillation is precipitated which persists even after stopping the stimulation (VFT). The normal rhythm is quickly restored by DC defibrillator. An increase in VFT indicates antiarrhythmic activity (M.Pande, K.Kar, M.Bhatnagar, M.P.Dubey and B.N.Dhawan, Supraspinal influences on ventricular fibrillation threshold and cardiac stores of norepinephrine in the cat, *Arch. int. Pharmacodyn.*, 225, 17, 1972). The procedure can be repeatedly performed in the same animal and is very useful for determining the duration of action. It does requires sophisticated equipment and experience and should be undertaken only after the isolation of the active principle.

5. Injury-induced Arrhythmias: Cats have to be used for these studies, which should be performed only with compounds selected for preclinical development. Myocardial infarction is produced in anaesthetised cat by ligating the anterior descending branch of left coronary artery in the 4th-5th left intercostal space with strict aseptic precautions. The ligation is done in two steps over 5-10 min period to prevent ventricular fibrillation. Initial arrhythmias soon disappear and are followed by persistent ectopic beats 24 hours later. This chronic unanaesthetised animal can be used repeatedly and biochemical

parameters in the serum like LDH, CPK and free fatty acids can also be measured to provide additional indication of drug efficacy (M.Pande, M.Bhatnagar, V.C.Pandey, K.Kar and B.N.Dhawan, Effect of antiarrhythmic drugs on serum free fatty acid levels during experimental cardiac arrhythmias in the cat, *Pharmacol.Res.Commun.*, 15, 100, 1983). Recently, similar acute procedure has been developed for anaesthetised rat (C.Clark, M.I.Foreman, K.A.Kane, F.M.McDonald and J.R.Parratt, Coronary artery ligation in anaesthetised rats as a method for the production of experimental dysrhythmias and for the determination of infarct size, *J.Pharm.Methods*, 3, 357, 1980) but the model has not been used extensively for new drug development.

D-HYPOLIPIDAEMIC ACTIVITY

Hyperlipidaemic disorders being a major cause of coronary artery disease and cerebrovascular accidents, there is a global interest in developing more effective and better tolerated hypolipidaemic agents. The interest in natural products has increased since CDRI introduced a standardised extract of *Commiphora mukul* as a new drug (B.N.Dhawan, Le Gugulipide, *Proc.Europhyto.*, 27, 1990) and with the data on several other natural products like Fenugreek seeds (R.D.Sharma, T.C.Raghuram and V.D.Rao, Hypolipidaemic effect of Fenugreek seeds. A clinical study. *Phytother.Res.*, 5, 145, 1991), solasodine (V.P.Dixit, M.Varma, N.T.Mathur, R.Mathur and S.Sharma, Hypocholesterolaemic and antiatherosclerotic effects of solasodine ($C_{24}H_{42}O_2N$) in cholesterol fed rabbits, *Phytother.Res.*, 6, 270, 1992), berginin (M.A.F.Jahram, J.P.N.Chansouria and A.B.Ray, Hypolipidaemic activity in rats of berginin, the major constituent of *Fluggea microcarpa*, *Phytother.Res.*, 6, 180, 1992) etc. published therafter. The assessment of the activity is primarily based on dependable biochemical estimations and good facilities for these determinations as well as satisfactory arrangement for long term maintenance of animals are absolute prerequisites. The *in vitro* techniques are more suitable for analysing the mechanism of action rather than for establishing the activity *per se*, and therefore receive a lower priority.

1. Triton-induced Hyperlipidaemia: This method introduced in 1955 (I.D.Frantz and B.T.Hinkelman, *J.Exp.Med.*, 101, 225, 1955), is an excellent acute method for initial screening. Adult male rats are given a single i.p. injection of Triton WR1339 (400 mg/kg) and the test extract (200 mg/kg) given orally. The animals are sacrificed 18-20 hours later. Blood cholesterol (A.Zlatkis, B.Zak and A.J.Boyle, A new method for direct determination of serum cholesterol, *J.Lab.Clin.Med.*, 41, 486, 1953), phospholipids (D.B.Zilversmit and A.K.Davis, Microdetermination of plasma phospholipid by trichloroacetic acid precipitation method, *J.Lab.Clin.Invest.*, 35, 155, 1950) and triglycerides (E. Van Handel and D.B.Zilversmit, Micromethod for direct determination of serum triglycerides, *J.Lab.Clin.Med.*, 50, 152, 1957) are determined. A lowering of 30% is considered for further follow-up. A higher or lower dose may need to be tested.

2. Lipid Level in Normal Animals: The test product is fed to male rats in a dose of 100 mg/kg (or 1/2 of the effective dose in the Triton test) for 30 days. Blood is collected every week and cholesterol, phospholipids and triglycerides are determined as described above. Even 20% lowering in normal animals is considered significant.

3. Diet-induced Hyperlipidaemia: Male rat are given a high fat diet of the following composition:

Constituent	%
Protein (Casein)	20
Cholesterol	5
Cholic acid	1.5
Fat (Hydrogenated vegetable oils)	15
Vitamin mixture	1
Salt mixture	0.5
Carbohydrate (Starch)	57

After being fed on this diet for two weeks the animals are given the drug for two weeks in the doses indicated for normal animals above. Blood is collected on 0,7 and 14th day of drug treatment and analysed for various lipid constituents as described above. Products giving 30% reduction should be processed further (S.Nityanand and N.K.Kapoor, Hypolipidaemic effect of ethylacetate fraction of *Commiphora mukul* (gugul) in rats, *Ind.J.Pharmacol.*, 6, 106, 1965). In these and other studies clofibrate or gugulipid (100 mg/kg) can be used as the standard drugs for comparison.

4. Propylthiouracil-induced Hypercholesterolaemia: Administration of 0.01% propylthiouracil in drinking water for 10 days produces 15% increase of serum cholesterol in male rats without any significant thyroid hypertrophy. This elevation should be prevented by concomittant administration of test agent (R.A.Turner, Screening Methods in Pharmacology, Academic Press, New York, p.260, 1965).

5. Follow-up Studies: These studies are undertaken with products selected for clinical development and are directed towards observing drug effects on atheromatous lesions, platelet function and analysing mechanism of action.

i.Effect on atheromatous lesions: Rabbits and monkeys, rendered hyperlipidaemic as described with rats, are used and the period of feeding is 8-12 weeks. A complete lipid profile is determined including fractionation of serum lipoproteins into VLDL, LDL (W.T.Friedwald, R.I.Levy and D.S.Fredrickson, Estimate of the concentration of low-density lipoprotein cholesterol in plasma without the use of preparative centrifuge, *Clin.Chem.*, 18, 499, 1972) and HDL (G.R.Warnick and J.J.Albers, A comprehensive evaluation of the heparin-manganase precipitation procedure for

estimating high-density lipoprotein cholesterol, *J.Lipid Res.*, 19, 65, 1978) and polyacramide disc gel electrophoresis for lipoprotein pattern. Progression and regression of atheromatous lesion is studied with light and electron microscopy.

ii.Inhibition of platelet aggregation: Platelet rich plasma (PRP, $3x10^8$ platelets/ml) is prepared from hypercitrated rabbit, monkey or human blood and 0.25 ml diluted to 0.275 ml with buffered (pH 7.4) saline. The test product ($1\text{-}5x10^{-4}$ M) is added, incubated for 3 min at 37°C and stirred for 2 min. Aggregation is than induced by ADP ($1x10^{-5}$ M), 5-HT ($1x10^{-4}$ M) or adrenaline ($1.2x10^{-4}$ M) and measured by an aggregometer. A reduction of more than 30% should be considered significant (L.Mester, M.Mester and S.Nityanand, Inhibition of platelet aggregation by gugul steroids, *Planta Med.*, 37, 367, 1979). The method is not suitable for crude materials.

iii.Isoprenaline-induced myocardial necrosis: There is an increase in some lipid constituents and superoxides in this rat model (G. Rona et al., An infarct like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat, *Arch.Path.*, 67, 443, 1959) which are prevented by pretreatment with hypolipidaemic agents.

iv.Antilipolytic action *in vitro*: Fat cells can be isolated from the epidydimal fat of normal and drug treated (as described under 2 above, for 14 days) male rats (M.Radbell, *J.Biol.Chem.*, 293, 375, 1944). One hundred mg fat cells are incubated in 3.0 ml Krebs-Ringer buffer (pH 7.4) containing 10 μg/ml adrenaline and 4% bovine serum albumin in metabolic shaker at 37°C for two hours. The free fatty acids (FFA) are then estimated (F.S.Mosinger, *J.Lipid Res.*, 6, 157, 1965). The inhibition of adrenaline-induced FFA release (μmol FFA/g fat cells/hour) indicates the antilipolytic activity of the test compound.

v.*In vitro* cholesterol biosynthesis: Liver homogenate (10%) from male rats is prepared in 0.1 M phosphate buffer (pH 7.4) with 0.25 M sucrose and cold centrifuged at 2500 rpm to remove unbroken cells and nuclei. The incubation mixture contains 2.0 ml homogenate, 0.03 M nicotinamide, 0.0016 M NAD, 0.001 M EDTA, 0.04 M $MgCl_2$ and 1 μCi/M ^{14}C-sodium acetate. The test compound is added in concentration of $0.2\text{-}0.4x10^{-3}$ g/ml and the mixture incubated at 37°C in a metabolic shaker for 90 min. The sediment is then saponified with 10% alcoholic KOH, the unsaponified fraction is extracted twice with petroleum ether. The cholesterol is precipitated as digitonoid and radioactivity of ^{14}C labelled cholesterol counted (N.K.Kapoor and S.Nityanand, *Ind.J.Biochem.Biophys.*, 15, 75, 1978).

vi.Other studies: Experiments of more specialised nature may be needed to fully elucidate the mechanism of action, e.g., interaction with LDL receptor from hepatocyte membrane, fecal excretion of cholesterol, lipid levels in liver, brain etc. requiring special experience and facilities. These will not be discussed here.

E-ANTITHROMBOTIC ACTIVITY

This has become an important area for new drug development due to the alarming increase in the incidence of thrombotic cardiovascular disease associated with high mortality and morbidity. Basically, the incident is associated with a diseased or damaged endothelium and animal models tend to simulate this by combining endothelial damage with stasis of blood and usually with the addition of clotting activator (arachidonic acid, collagen, PAF). Effect of drug is also studied on platelet function and other thrombotic factors. Most of the experimental procedures have been developed during the last ten years and so far have had rather limited application to drug research. Curcumin, obtained from rhizomes of *Curcuma longa*, appears to be only lead from the natural products so far.

1. Collagen- and Adrenaline-induced Pulmonary Thromboembolism in Mice: Male adult mice are treated with the test compound by i.p. or oral route in groups of five animals each. One hour later 15 mg collagen and 1.8 μg adrenaline in 0.1 ml normal saline are injected intravenously via the tail vein. Unprotected animals get hind limb paralysis or die (G.DiMino and M.G.Silver, Mouse antithrombotic assay: A simple method for the evaluation of antithrombotic agents *in vivo*. Potentiation of antithrombotic activity of ethyl alcohol, *J.Pharmacol.Exp.Ther.*, 225, 57, 1983).

2. Production of Venous Thrombosis: Male adult rats are used and thrombus can be formed in conscious or anaesthetised animals. In both cases abdominal inferior vena cava is used.

i.Conscious animal: Under ether anaesthesia a small stainless steel coil is inserted into the inferior vena cava just below the junction with the left renal vein and the animal allowed to recover. The coil is removed 48 hours later and the amount of thrombus assessed by determining its protein content (O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, Protein measurement with Folin phenol reagent, *J.Biol.Chem.*, 193, 265, 1951). Effective agents reduce the amount of the thrombus formed.

ii.Stasis-induced thrombosis: The inferior vena cava is isolated under ether anaesthesia and a ligature of cotton thread tied below its junction with the left renal vein and the abdomen is closed. The abdomen is reopened three hours later under ether anaesthesia, the vena cava clamped two cm below the ligature and the section between the clamp and the ligature cut longitudinally to remove the thrombus which is formed in 97% of untreated animals. The thrombus is dried for 24 hours and its weight determined in a sensitive balance (A.Bernat, E.Vallee, J.P.Maffrand and R.Roncucci, Antithrombotic effect by Ticlopidine in a platelet-independent model of venous thrombosis, *Thromb.Res.*, 37, 279, 1985). The difference in mean thrombus weight between the treated and control animals is tested for statistical significance.

3.Extracorporeal Shunt: This method has a wide applicability ranging from the rat (T.Umetsu and K.Sanai, Effect of 1-methyl-2-mercapto-5-(3-pyridyl)-imidazole (KC-6141), an antiaggregating compound on experimental thrombosis in rats, *Thromb.Haemostas.*, 39, 74, 1978) to guinea-pig, rabbit,

dog and even baboon. Under pentobarbitone anaesthesia the animals are heparinised (100 U/kg i.v.) and a series of three polythene tubes inserted between the right carotid artery and the left jugular vein to establish the extracorporeal shunt. The middle tube is 6 cm long, filled with heparin solution (100 U/ml) and contains a 5 cm long white silk thread. The tubes at its two end are 12.5 cm long and also contain heparinised saline. After 20 min the vessels are clamped and the thread removed to get the weight of wet thrombus. Repeated acute experiments can be performed in the same animal and at least 2-3 control values must be obtained before drug administration through a cannulated vein. The procedure is repeated after drug administration and, in a larger animal, several doses can be tested to establish a dose-response relationship.

4.Prevention of Arachidonate-induced Mortality: Male rats are anaesthetised with pentobarbitone and 5-20 mg sodium arachidonate injected via the left common carotid artery. The unprotected animals die within 60 min. Drug treatment is given half an hour earlier and indomethacin or aspirin can be used as standard drug for comparison (K.Sakai, T.Yamazaki, Y.Nakamura and T.Sugimoto, Protective effects of antianginal agent microandil on arachidonate-induced sudden death: Comparison with several anti-anginal agents and cyclooxygenase inhibitors, *Jap.J.Pharmacol.*, 37, 124, 1985).

5.Effect on Platelet Function: Attempts have been made to study platelet function *in vivo*, for example, by monitoring ECG alteration and platelet count following ADP infusion (E.Dejana, M.G.Castell, G.DeGaetano and A.Bonaccorsi, Contribution of platelets to cardiovascular effects of ADP in rat, *Thromb.Haemostas.*, 39, 135, 1978). These methods, however, have not found a general acceptance. Inhibition of platelet aggregation is still extensively used and preferred. The experiments can be performed *in vitro* (Details in section on Hypolipidaemic Activity) or *ex vivo* by preparing platelet rich plasma (PRP) from drug treated animals (B.N.Dhawan, M.Pande, R.C.Srimal and R.Srivastava, Antithrombotic effect of curcumin, *Thromb.Res.*, 40, 413, 1985). Further studies are undertaken with the active compounds to study their effects on prostaglandin and prostacyclin synthesis (R.Srivastava, V.Puri, R.C.Srimal and B.N.Dhawan, Effect of curcumin on platelet aggregation and prostacyclin synthesis, *Arzneim. Forsch.*, 36, 715, 1986; R.Srivastava, V.Puri, R.C.Srimal and B.N.Dhawan, Prostanoid mediated effects of centchroman, a nonsteroidal oral contraceptive, *Agents and Actions*, 18, 596, 1986).

6.Effect on Other Thrombotic Factors: These detailed studies are required only in the case of pure or nearly pure materials selected for preclinical development. They evaluate the interaction of the drug with various plasma factors by determining the fibrinolysis (T.Astrup and S.Mullerts, The fibrin plate method for estimating fibrinolytic activity, *Arch.Biochem.Biophys.*, 40, 345, 1952), euglobulin utilisation (E.A.Copley and S.Niewiarovski, A micro method of euglobulin fibrinolysis in plasma of human subjects and laboratory animals,J.Clin.Med., 53, 468, 1959) and effect on tissue plasminogen activator (TPA), for which EIA kit is available commercially, and other activators (T.H. Carter, B.A.Everson and O.D.

Ratnoff, Cabbage seed protease inhibitor, a slow tight binding inhibitor of trypsin with activity toward thrombin, activated Stuart factor (Factor XA), activated Hageman Factor (Factor XIIA) and plasmin, *Blood*, 75, 108, 1990).

Annexure VIII

HEPATOPROTECTIVE ACTIVITY OF NATURAL PRODUCTS - EXPERIMENTAL EVALUATION

B.N. Dhawan
Central Drug Research Institute
Lucknow 226001, INDIA

There has been increasing interest in this area because of several reasons. No drug from synthetic source has been developed so far and none appears even on the horizon. Infective hepatitis is widespread and it is realised that the sequale may not be only a chronic carrier stage but also subacute hepatitis leading to cirrhosis or hepatic malignancy. Finally there is an alarming increase in the incidence of alcohol related liver damage. Many plant products are used in traditional systems of medicine in various countries, other plants enjoy folklore reputation. There have been recent reviews of the global (S.S. Handa, A. Sharma and K.K. Chakrobarti, Natural products and plants as liver protective drugs, *Fitoterapia*, 57, 307, 1986) as well as regional (Y. Ozturk, K.H.C. Baser and S. Aydin, Hepatoprotective (antihepatotoxic) plants in the Turkey, In: K.H.C. Baser, Ed. Proc. 9th Symp. on Plant Drugs, Eskisehir, p.223, 1991) status of these plants but the only product available for modern practioners is silymarin obtained from seeds of *Silybum marianum* (H.M. Raven and H. Schriewer, Die antihepatotoxische Wirkung von Silymarin bei experimentalellan - leberschädigungen der rätte durch tetrachlorokohlenstoff, D-galaktosamin und allylalkohol, *Arzneim Forsch.*, 21, 1194, 1971). Our recent work on *Picrorhiza kurroa* (R.A. Ansari, B.S. Aswal, R. Chander, B.N. Dhawan, N.K. Garg, N.K. Kapoor, D.K. Kulshrestha, H. Mehdi, B.N. Mehrotra, G.K. Patnaik and S. K. Sharma, Hepatoprotective activity of Kutkin-the iridoid glycoside mixture of *Picrorhiza kurroa*, *Ind.J.Med.Res.*, 87, 401, 1988) has led to clinical studies with a standardised iridoid glycoside fraction named Picroliv. Among other promising active principles mention may be made of n-demethyl ricinine (B. Shukla, P.K.S. Visen, G.K. Patnaik, N.K. Kapoor and B.N. Dhawan, Hepatoprotective activity of an active constituent isolated from the leaves of *Ricinus communis* Linn., *Drug Develop.Res.*, 26, 183, 1992), andrographolide (isolated by Boorsma in 1896) from *Andrographis paniculata* (Handa et al, cited above) and coumestans (H. Wagner, B. Geyer, Y. Kisi, H. Hikino and G.R. Rao, Coumestans as the main active principles of the liver drugs *Eclipta alba* and *Wedelia calendulacea*, *Planta Med.*, 52, 370, 1986).

The multifarious activities of the liver may be conveniently grouped under secretory (glycogen, cholesterol, lipids etc), excretory (bile salts and acids), metabolic (drug detoxification) and immunological. A comprehensive screening for hepatoprotective agents should, therefore, evaluate all these activities. The large variety of screening procedures available can be divided into 3 groups depending upon the activity they detect:

1. Antagonism of the effect of hepatotoxic agents
2. Choleretic and anti-cholestatic effects
3. Immunomodulator activity

Over the last 10 years CDRI has developed a composite screen to study these activities and this has been largely instrumental in uncovering the unique spectrum of activity of Picroliv. It can be profitably adopted by any laboratory interested in new hepatoprotective agents with such modifications as local conditions warrant. The CDRI protocol has the advantage of undertaking most of the testing in rat and mice and placing only limited reliance on *in-vitro* techniques, which do have limitations, specially when very crude products are to be tested.

A. ANTAGONISM OF HEPATOTOXINS

These experiments essentially measure the prophylactic efficacy of the hepatoprotective agents against a battery of hepatotoxins, mainly because of the acute nature of toxicity of most toxins. In some case a curative activity can also be evaluated. The toxins can affect various organelles of hepatocytes, it is therefore necessary to include markers for various subcellular elements to get a correct assessment. Morever, various toxins have different target sites within the hepatocytes, so several hepatotoxins have to be included. Carbon tetrachloride and galactosamine have been most extensively used. We have found it necessary to utilise a much large number of toxins. The agents used, along with their doses and duration of experiment are summarised in Table 1. Rats are used unless otherwise mentioned.

TABLE 1: THE HEPATOXICANTS USED IN CDRI SCREENS

Hepatoxicant	Dose 1 kg	Route	Sacrifice after (days)
1. Aflatoxin B	7 mg	oral	4
2. *Amanita phalloides*	50 mg	oral	10
3. Carbon tetrachloride	0.7 ml x 6 on alt. day	i.p.	2
4. d-Galactosamine *	8.00 mg	i.p.	1
5. Lanthanum	4 mg	i.v.	4
6. Monocrotaline (Pyrrolizidine Alk.)	120 mg	oral	12
7. Paracetamol *	2.0 g	oral	2
8. Plasmodium berghei	5×10^5 parasitised RBC	i.p.(mastomys)	15
9. Thioacetamide *	100 mg	subcut.	2

* Primary screens

It has been seen that in most cases optimum effect is obtained if the protective agent is given for 8-10 days, hence suitable pretreatment is needed so that the last dose of the test agent is given 24 hours before the sacrifice. The protective effect can be assessed most comprehensively by biochemical estimations on the liver and the serum. Additionally, histopathological procedures, indirect assessment of the activity of drug metabolising enzymes and *in vitro* studies in hepatocytes have been used.

1. Assessment by Biochemical Parameters: As already discussed above, it is necessary to employ a large number of parameters. The parameters employed, the information provided by them and the methods of their estimation have been summarised in Tables 2 (Hepatic enzymes), 3(Other hepatic parameters) and 4(Serum parameters) along with the range of normal values obtained at CDRI.

It is not possible to provide details of all these estimations due to constraint of space, but it will be observed that most require simple biochemical facilities and for many of these semiautomatic assay instruments with chemical kits are available. About 75% of the liver tissue and 5 ml blood are adequate for all the determinations.

The major changes observed in these parameters with various hepatotoxic agents have been summarised in Tables 5-7 and this will clearly demonstrate the need of using so many parameters as well as a battery of hepatotoxicants (each with its unique spectrum of changes) to get dependable data about hepatoprotective efficacy. We have used all these procedures extensively in our recent studies and a list of relevant publications is given in Appendix I.

TABLE 2: MARKER ENZYMES FOR HEPATIC FUNCTION, THEIR NORMAL VALUES AND ASSAY METHODS

Enzyme (EC No)	Marker	CDRI Range (Units/100 mg protein)	Method of determination
1. Succinate Dehydrogenase (1.3.99.1)	Mitochondria	0.73-1.08	E.C.Slater and W.D. Bonner. The effect of fluoride on the succinate oxidase system. *Biochem.J.*, 52, 185, 1952
2. Glucose-6-Phosphatase (3.1.3.9)	Mitochondria	8-9.7	G. Hubscher and G.R. West. Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. *Nature*, 205, 799, 1965
3. Cytochrome P_{450} * (1.14.14.1) b_5 * (1.6.2.2)	Microsome	24.9-39.9 25.2-27.5	T. Omura and R. Sato. The carbon monoxide binding pigment of liver microsomes I. evidence for its hemoprotein nature. *J.Biol.Chem.*, 239, 2370, 1964
4. 5'-Nucleotidase (3.1.3.5)	Plasma Membrane	3-4.21	N.N. Aronson Jr. and O. Touster. Isolation of rat liver plasma membrane fragments in isotonic sucrose. In: S. Fleisher and L. Packer Eds. *Methods in Enzymology*, 31, 90, 1974
5. γ -Glutamyltranspeptidase (2.3.2.2)	Plasma Membrane	43.4-50.5	V. Boelsterli and G. Zbinden. Application of fine needle aspiration biopsy for the diagnosis of dysplastic and neoplastic liver cell changes induced by N-nitrosomorpholine in rats. *Arch.Toxicol.*, 42, 225, 1979
6. Acid Phosphatase (3.1.3.2)	Lysosomal Fraction	5.68-6.25	P.J. Wright, P.D. Leathwood and D.T. Plumner. Enzymes in rat urine. Acid Phosphatase. *Enzymologia*, 42, 459, 1972
7. Acid Ribonuclease (3.1.27.5)	Lysosomal Fraction	4.16-5.71	C.de Duve, B.C. Pressman, R. Gianetto, R. Wattiaux and F. Applemans. Tissue fractionation studies .6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J.*, 60, 604, 1955
8. Superoxide dismutase * (1.15.1.1)	Post-mitochondrial Fraction	2.52	J.M. McCord and I. Fridovich. Superoxide dismutase: an enzyme function for erythrocuprein (Hemocuprein). *J.Biol.Chem.*, 244, 6049, 1969

* Cold centrifugation at 12.000-150.000 g required

TABLE 3: OTHER MARKERS FOR LIVER FUNCTION WITH THEIR RANGE AND ASSAY PROCEDURES

Parameter	Marker	CDRI Range (mg/g liver)	Method of determination
1. Proteins	Membrane Constituent	132-158	O.H. Lowry, N.J.Rosebrough, A.L. Farr and R.J. Randall. Protein measurement with Folin phenol reagent. *J.Biol.Chem.*, 193, 265, 1951
2. Phospholipids	Membrane Constituent		H. Wagner, A. Lissau, J. Holzl and L. Horhammer. The incorporation of P_{32} in to the inositol phosphatides of rat brain. *J.Lipid Res.*, 3, 177, 1962
3. Cholesterol	Membrane Constituent	7.8-12.0	A. Zlatkis, B. Zak and A.J. Boyle. A new method for the direct determination of serum cholesterol. *J.Lab.Clin.Med.*, 41, 486, 1953
4. DNA	Hepatocellular repair	2.00-2.76	Z. Dische. Some new characteristic color tests for thymonucleic acid and a microchemical method for determining the same in animal organs by means of these tests. *Mikrochemie*, 8, 4, 1930
5. RNA	Hepatocellular repair	3.56-4.99	W. Mejbaum. Estimation of small amounts of pentose especially in derivatives of adenylic acid. *Z.Physiol.Chem.*, 258, 117, 1939
6. Glycogen	Hepatocellular damage	40.22-62.70	R. Montgomery. Determination of glycogen. *Arch.Biochem.Biophys.*, 67, 378, 1957
7. Total lipids	Disturbed lipoidosis	32.3-50	J. Folch. M. Lees and G.H.Sloane Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J.Biol.Chem.*, 226, 497, 1957
8. Lipid Peroxides	Disturbed lipoidosis	145.8	H. Ohkawa, N. Ohishi and K. Yagi. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal.Biochem.*, 95, 351, 1979
9. Lipoprotein X	Disturbed lipoidosis	Not present normally	E. Talafant and J. Tovarek. Rapid colorimetric methods for quantitative lipoprotein-X determination without electrophoresis. *Clin.Chem.Acta.*, 88, 215, 1978

TABLE 4: SERUM PARAMETERS USEFUL AS INDICATORS OF LIVER FUNCTION

Parameter in Serum	CDRI Range	Reference for determination
1. Glutamic oxaloacetate transaminase (E.C. 2.6.1.1)	52-135μmol oxaloacetate/hr/L	S. Reitman and S. Frankel. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am.J.Clin.Pathol.*, 28, 56, 1957
2. Glutamic pyruvic transaminase (E.C. 2.6.1.2)	52-112μmol pyruvate/hr/L	
3. Glutamic dehydrogenase (E.C. 1.4.1.2)	1.37-3.88μmol oxoglutarate hr/L	J. King. Glutamate dehydrogenase: Colorimetric assay In: H.V.Bergmeyer Ed. Methods in Enzymatic Analysis. Academic Press. New York, p.656, 1963
4. Alkaline phosphatase (E.C. 3.1.3.1)	27-61μmol p-nitrophenol released/min/dL	O.A.Bessey, O.H. Lowry and M.J. Brock. A method for the rapid determination of alkaline phosphatase with five cubic milimeters of serum. *J.Biol.Chem.*, 164, 321, 1946
5. Total proteins	5.2-8.6 g/dL	O.H. Lowry, M.J.Rosebrough, A.L.Farr and R.J.Randall. Protein measurement with Folin phenol reagent. *J.Biol.Chem*, 193, 265, 1951
6. Albumen	3.2-3.38 g/dL	R. Stavric-Hirosho, V. Arsova and A. Pop-Stefanova. Determination of albumin in serum and urine with bromocresol green. *Anal.Abs.*, 26, 2818, 1974
7. Bilirubin	0.15-0.43 mg/dL	E. Malloy and K.Evelyn. The determination of bilirubin with the photoelectric colorimeter. *J.Biol.Chem.*, 119, 481, 1937
8. Lipoprotein-X	not present normally	E. Talfant and J. Tovarek. Rapid colorimetric methods for quantitative lipoprotein-X determination without electrophoresis. *Clin.Chem.Acta.*, 80, 215, 1958

TABLE 5: CHANGES IN LIVER ENZYMES BY HEPATIC TOXINS

ENZYME	DECREASE	INCREASE
1. Succinic dehydrogenase	Carbon Tetrachloride Paracetamol *P. berghei*	*A. phalloides* Lanthanum Pyrrolizidine alkaloids
2. Glucose-6-Phosphatase	*A. phalloides* Carbon Tetrachloride Paracetamol *P. berghei* Thioacetamide	None
3. Acid-Phosphatase 4. Acid-Ribonuclease 5. 5-Nucleotidase 6. γ-Glutamyl Transpeptidase	None	All
7. Superoxide Dismutase	Carbon Tetrachloride *P. berghei*	None
8. Cytochrome P_{450}	All	None
9. Other Drug Metabolising Enzymes	All	None

TABLE 6: CHANGES IN OTHER HEPATIC CONSTITUENTS BY TOXINS

PARAMETER	DECREASE	INCREASE
1. Total Lipids	None	All except Thioacetamide
2. Lipid Peroxides	None	All except Paracetamol and Thioacetamide
3. Cholesterol	None	All except Pyrrolizidine alkaloids and Thioacetamide
4. Phospholipids	None	Paracetamol
5. Glycogen	All	None
6. Proteins	Pyrrolizidine alkaloids	Carbon Tetrachloride Paracetamol Thioacetamide
7. DNA	Pyrrolizidine alkaloids	Galactosamine Thioacetamide
8. RNA	Pyrrolizidine alkaloids	Thioacetamide

TABLE 7: SERUM CONSTITUENTS AFFECTED BY HEPATOTOXIC AGENTS

CONSTITUENT	DECREASE	INCREASE
1. SGPT 2. SGOT	None	All except Lanthanum
3. Alkaline Phosphatase	None	All except *A. Phalloides* Paracetamol Pyrrolizidine alkaloids
4. Glutamate Dehydrogenase	None	All except *A. Phalloides* *P. berghei*
5. Bilirubin	None	All except *A. phalloides*
6. Cholesterol	Galactosamine	*P. berghei*
7. Triglycerides	None	Carbon Tetrachloride
8. Lipoprotein-X	None	Carbon Tetrachloride *P. berghei*
9. Proteins	*P. berghei* Pyrrolizidine alkaloids	Carbon Tetrachloride
10. Albumen	*A. Phalloides* Galactosamine Lanthanum	*P. berghei*

2. Histopathological Studies: Several small pieces from each lobe of liver should be taken and fixed in 10% buffered formalin. After routine processing and paraffin embedding 4μm serial sections are cut and stained by haematoxylin eosin. For reticulin Wilder's silver impregnation is used. It is necessary to examine a large number of slides from each lobe, preferably by 2 independent observors who are unaware of the treatment given to rats, before drawing conclusions. Histopathological studies usefully complement results from biochepmical studies described above and should best be used in conjunction with them. Histopathological studies are very useful in studying drug effects on liver regeneration. Partially hepatectomised animals are used in such studies and besides routine histopathology molecular biology procedures like DNA hybridisation should be used concurrently.

3. Activity of Drug Metabolising Enzymes: The activity of these enzymes is decreased by all hepatic toxicants (see Table 5). A direct estimate is obtained by assay of Cytochrome P_{450} or b_5 as described above but their activity can also be indirectly judged by monitoring blood concentration of agents metabolised by liver following a standard dose in normal, toxicant treated and toxicant + hepatoprotective agent treated groups. Any other effect of hepatoprotective agent e.g. on rate of absorption of the assayed compound must be excluded. A commonly used method is studying the effect on barbiturate narcosis, since it does not involve any biochemical estimation. Sleep induced by a liver-metabolised barbiturate like hexobarbitone (75 mg/kg/ip) is increased by hepatic toxicants and

the increase is prevented by hepatoprotective agents (Y. Ozturk, S. Aydin, K.H.C. Baser, N. Kirimer and N.Kurtar-Ozturk, Hepatoprotective activity of *Hypericum perforatum* L. alcoholic extract in rodents, *Phytotherapy Res.*, 6, 44, 1992). Several plant extracts have a CNS effect of their own and can thus affect the duration of barbiturate hypnosis. If a CNS effect is present it is useful to include another group treated with a barbiturate not metabolised by liver, like barbitone (125 mg/kg ip) for comparison.

4. Studies on Isolated Hepatocytes: These studies can be *in vitro* or *ex vivo*. In former case isolated hepatocytes are incubated with varying concentration of the test drug, in the later hepatocytes are isolated from drug treated animals and their viability and other parameters studied. The later method is particularly suitable for crude extracts.

i. Isolation of hepatocytes: Hepatocytes are isolated from ether anaesthetised adult rats or guinea pigs. After laprotomy, the animal is heparinised by intracardiac injection of 1000 units (0.2 ml) heparin. The inferior vena cava (near kidney) and distal end of portal vein are ligated and the later is cannulated proximal to the ligation with a fine polyethylene cannula. The liver is perfused via the cannula, initially with oxygenated calcium free buffer (sodium chloride 0.142M, potassium chloride 0.0067M, HEPES 0.01M in distilled water, pH7.4, temperature 37°C) at a flow rate of 2.5-3 ml/min/g liver for 12-15 minutes via a peristaltic pump, a cut is made at the upper end of inferior vena cava for the fluid to escape. As the blood is washed away the liver becomes greyish brown. It is now perfused with the same buffer with 0.1 m HEPES and 1.6×10^{-9} g/ml collagenase (630 units/mg, Sigma) and pH7.6 at the same rate for 7-8 minutes (P.O. Seglen, Preparation of isolated liver cells, *Methods Cell Biol.*, 13, 29, 1975). We have introduced some modification to conserve the amount of collagenase by reperfusion (P.K.S. Visen, B. Shukla, G.K. Patnaik, S. Kaul, N.K. Kapoor and B.N. Dhawan, Hepatoprotective activity of picroliv, the active principle of *Picrorhiza kurroa* on rat hepatocytes against paracetamol toxicity, *Drug Develop.Res.*, 22, 209, 1991). The liver swells and it is now removed, washed with HEPES buffer and gently teased to disperse the cells after disrupting the surrounding Glisson's capsule. The cells are dispersed in Leibovitz dispersal medium (L_{15}), filtered through a muslin cloth to remove cell debris etc., washed thrice with cold medium and centrifuged at slow speed (200 rpm for one minute). The pellet is placed in L_{15} medium for further study.

ii. Viability Tests: The most commonly used test is Trypan Blue exclusion test. Dye is added in 0.2% concentration and a microscopic examination shows the damaged cells to be stained. A preparation with less than 10% damaged cell can be obtained with experience. Treatment with hepatotoxic agents reduces the percentage of viable cells significantly. The staining method alone is not considered adequate to judge the normal functioning of hepatocytes. Hence their oxygen uptake is also studied using a Gilson's oxygraph (J.E. Eastbrook, Mitochondrial respiratory control, polarographic measurement of ADP: O ratio, *Methods Enzymol.*, 10, 41, 1967) and 0.1 ml cell suspension in 1.7 ml HEPES buffer. In addition to studying normal oxygen uptake, increased oxygen uptake after adding different substrates like ADP,

succinate, succinate + ADP or glucose should also be studied. In each case the effect of hepatotoxins and its reversal by test drug is studied (P.K.S. Visen, B. Shukla, G.K. Patnaik, R. Chandra, V.Singh, N.K. Kapoor and B.N. Dhawan, Hepatoprotective activity of picroliv isolated from *Picrorhiza kurroa* against thioacetamide toxicity on rat hepatocytes, *Phytotherap.Res.*, 5, 224, 1991).

iii. Biochemical Parameters: A number of biochemical parameters can be measured in the isolated hepatocytes to monitor the effect of the toxins as well as of hepatoprotective agents. These include enzymes like the transaminases and alkaline phosphate and other constituents like cholesterol, RNA, DNA, bile salts, cholic acid and deoxycholic acid. The bile salts can be estimated by the method of Hawk et al (P.B. Hawk, B.L. Oser, and W.H. Summerson, Bile and liver function, In: P.B. Hawk ed. **Practical Physiological Chemistry**, McGraw Hill Book Co. Inc., New York, p.408, 1954) and the bile acids by the procedure of Erwin et al (H. Erwin, H. Mosbach, E.H. Klenisky and E. Forrest, Determination of deoxychoiic acid and cholic acid in bile, *Arch.Biochem.Biophys.*, 51, 402, 1954). The methods for estimation of other constituents have been given in Tables 3 and 4 respectively. The normal values of various paramet rs are expressed in relation to protein content (determined by the method of Lowry et al (cited above) and have been shown in Table 8. The standard serum parameters should also be monitored in case of *ex-vivo* studies. For *in-vitro* studies hepatocytes from normal animals can be incubated with the toxin for upto 24 hours and then with the hepatoprotective agent for the same period in a CO_2 incubator at 37°C under 5% CO_2 to study the reversal of toxin induced changes. If prophylactic activity is to be investigated, the toxin and test compound are added together to the incubation medium and effects evaluated 24 hours later. The viability, oxygen uptake and various biochemical parameters are estimated in control, toxin treated and toxin with test drug treated hepatocytes. If proper temperature and nutritional conditions are provided the hepatocytes remain viable for 3-4 weeks in a CO_2 incubator.

TABLE 8: NORMAL VALUES OF SELECTED BIOCHEMICAL PARAMETERS IN RAT HEPATOCYTES

PARAMETER	CONCENTRATION/MG PROTEIN
GOT	12.8 Units
GPT	12.9 Units
Alkaline Phosphatase	1.6 Units
Cholesterol	1.6 μg
RNA	8.7 μg
DNA	0.6 μg
Bile Salts	4.3 μg
Cholic Acid	2.9 μg
Deoxycholic Acid	2.3 μg

5. Studies in Cultured Hepatocytes: The basic experimental design is similar to that used for isolated hepatocytes. The culture is best initiated with hepatocytes from fetal rat liver. Pregnant female rats are anaesthetised with ether on the 19th day, fetuses taken out and liver removed under aseptic condition in a laminar flow cabinet. It is washed with HEPES buffer, cut in small pieces and incubated with 0.2mg/collagenase in RPMI-1640 medium at 37°C for 45 minutes (G. Yeoh, Enzymes and plasma proteins in cultures of fetal hepatocytes, In: A. Guillouzo and C. Guguen-Guillouzo Eds. Research in Isolated and Cultured Hepatocytes, John Tibbey & Co.Ltd., London, p.171, 1986). The collagen treated liver is gently minced, filtered through muslin cloth and centrifuged at 200 rpm for one minute. The hepatocytes are washed thrice and finally resuspended in RPMI-1640. A viability test (Trypan blue) is performed before seeding them ($1x10^6$ cells per flask) in culture flasks containing RPMI-1640 with HEPES buffer supplemented with 10% calf serum and gentamycin. In a humidified CO_2 incubator hepatocytes attach to the substratum within 1/2-1 hour and within a few days monolayer is formed. The primary culture can be used as such, but if a secondary culture is desired, growth factors (Epidermal growth factor, Insulin like growth factor, Platelet derived growth factor), neo-natal calf serum and insulin have to be added. The medium employed is RPMI-1640 with L-glutamine and HEPES but without sodium carbonate. Further details may be obtained from Tingstrom and Obrink (A. Tingstrom and B. Obrink, Distribution and dynamics of cell surface-associated Cell CAM105 in cultured rat hepatocytes, *Exp.Cell.Res.*, 185, 132, 1989). We do not find any advantage in cultured hepatocytes over the isolated hepatocytes for study of hepatoprotective activity of natural products, on the contrary cultured hepatocytes require greater care and attention and are much more expensive to maintain.

B: CHOLERETIC AND ANTICHOLESTATIC ACTIVITY

These studies are undertaken in unanaesthetised (rat) or anaesthetised animals (rat, guinea pig) and evaluate the effect on the volume as well as constituents (bile salts and bile acids-cholic and deoxycholic) of the bile. The chemical methods of estimation of bile salts and bile acids have been described in a previous section (studies on isolated hepatocytes). The experiments are designed to measure the drug effect on normal flow (choleretic) and on cholestasis induced by ethinylestradiol (5 mg/kg subcut for 3 days, B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan, Choleretic effect of Picroliv, the hepatoprotective principle of *Picrorhiza kurroa*, *Planta Med.*, 57, 29, 1991), paracetamol (2g/kg oral, B. Shukla, P.K.S. Visen, G.K. Patnaik, N.K. Kapoor and B.N. Dhawan, Hepatoprotective effect of an active constituent isolated from the leaves of *Ricinus communis* Linn., *Drug Develop Res.*, 26, 183, 1992) or thioacetamide (B. Shukla, P.K.S.Visen, G.K. Patnaik and B.N. Dhawan, Reversal of thioacetamide induced cholestasis by Picroliv in rodents, *Phytother.Res.*, 6, 53, 1992). Similar studies could also be planned with other hepatotoxic agents. In addition, as pointed out in an earlier section, concentration of bile salts and bile pigments can also be measured in normal hepatocytes and those damaged by hepatotoxin *in vitro* or *ex vivo*.

To study the effects of a plant product on biliary secretion it is usually necessary to administer it for several days since the effect of a single dose is usually not very marked. In conscious animals the bile flow can be recorded daily and its contents analysed. The studies on anaesthetised animals are generally performed 24 hours after the last dose of the test drug and effects compared with bile flow on control animals and those treated with cholestatic agent only. For studies in conscious animals rats are used. Under light ether anaesthesia abdomen is opened by a midline incision, the common bile duct is exposed and cut, the proximal end is cannulated with a fine polyethylene tube which is pushed under the skin on the back and taken out between the ears. The abdomen is stitched, the tubing protected by a light iron spring and the animal allowed to recover for 1-2 days. The bile flow can be recorded for any length of time and expressed as ml/100g body wt/hr. For acute experiments anaesthetised rats (pentobarbitone 40 mg/kg ip), guinea pigs (urethane 6 ml/kg of 25% solution, ip, B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan, Choleretic effect of andrographalide in rats and guinea pig, *Planta Med.*, 58, 146, 1992) or cats (pentobarbiotone 40 mg/kg iv.) can be used

C. IMMUNOMODULATOR ACTIVITY

Immunomodulator activity is not present in all hepatoprotective agents but is a very useful adjunct to look for. It is beneficial to employ some simple tests initially which can be used with crude extracts as well and can be performed concurrently with other tests for hepatoprotective activity. The CDRI tests are performed in rat, Balb/c or conventional mouse and utilise the same routine of drug administration as used for hepatoprotective activity. Balb/c mice are better animals for these studies. The extract is administered orally (25 mg/kg crude extract, 10 mg/kg pure compound) for 7 days and on the 8th day

they are immunised with 10^8 sheep RBC (SRBC) by ip route and sacrificed 4 days later to collect the serum and spleen. For non-antigen mediated (non specific) response the animals are sacrificed on the 8th day and peritoneal exudate and spleen is collected. Non specific response can also be studied by observing protection against infection.

1. Antigen Specific Responses: Serum and spleen can be collected from the same group of animals but a separate groups is included for studying the delayed type of hypersensitivity (DTH). Three tests are routinely used.

i. Haemagglutinating antibody (HA) titre: The microtitre plate is used and 50 microlitre aliquots of two fold serial serum dilution are prepared in 0.1MNaCl containing 0.05M phosphate buffer (pH7.2). The saline is also used for making 1% SRBC suspension. 25 microlitre is added to each well, mixed and plates incubated at room temperature for 1½-2 hours before observing agglutination (P.R. Dua, G. Shankar, R.C. Srimal, K.C. Saxena, R.P. Saxena, A. Puri, B.N. Dhawan, Y.N. Shukla, R.S. Thakur and A. Husain, Adaptogenic activity of Indian *Panax pseudoginseng*, *Ind.J.Exp.Biol.*, 27, 631, 1989). The reciprocal of the highest concentration giving 50% agglutination is HA Titre.

ii. Plaque forming cell (PFC) assay: The technique of Jerne and Nordin (N.K. Jerne and A.A. Nordin, *Science*, 140, 405, 1963) is followed, the spleen cells ($1x10^6$ cells/ml) being suspended in RPMI-1640. In a petri-dish a bottom layer of 1.2% agarose in 0.15M NaCl is prepared and a mixture of 2 ml 0.6% agarose in RPMI-1640(42°C), 0.1 ml 20% SRBC and 10^5 spleen cell suspension poured over it and incubated at 35°C (90 minutes). Complement is then provided by adding 2 ml of 1:10 diluted fresh guinea pig serum and incubated for 45 minutes more. The plaques are counted and expressed as counts per 10^5 spleen cells (R.J. Mishell and R.W. Dutton, Immunisation of dissociated spleen cell cultures from normal mice, *J.Exp.Med.*, 126, 423, 1967).

iii. DTH response: The SRBC immunised mice are injected with $1x10^8$ SRBC in a hind foot pad (I. Saiki, Y. Tanio, M. Yamewake, M. Uemuja, S. Kabayasli, T. Fukuda, H. Yukimasa, Y. Yamamura and I. Axuma, *Infec.Immun.*, 31, 114, 1981) and the foot pad reaction measured 24 hour later either by its thickness (Schnelltester, Kroplin) or plethysmographically (e.g. Ugo Basile Assembly), the difference between the 2 hind foot pads indicating the response (C. Papadimitriou, H. Hahn, H. Waher and S.H.E. Kaufman, Cellular immune response to sheep erythrocytes, interrelationship between proliferation of popliteal lymph nodes and foot pad swelling, *Immunol.*, 164, 361, 1983). Similar studies can be performed in sensitised guinea pig (egg albumen 50mg/kg on days 1 and 3 and testing after 2-3 weeks); the effect being studied on the ileum, bronchi (*in vitro* or *in vivo*) or the conjunctiva (*in vivo*).

2. Non-specific Immune Responses: These responses are basically studied to investigate the effect on selected activities of macrophages and lymphocytes.

i. Macrophages: Macrophages can be collected from the peritoneum of sensitised mice or rats (egg albumen), by injecting normal saline, gentle massage and aspiration, in siliconised test tubes containing RPMI-1640 (pH 7.42). They are washed thrice by low speed centrifugation (400-500 rpm) and discarding the supernatant and suspending the pellet in complete RPMI-1640 medium containing 10% fetal calf serum. For phagocytic activity one ml aliquots containing 1x 10^6 cells are incubated at 37°C in 16 well cluster plates in a CO_2 incubator (5% CO_2) for 60 minutes and the monolayer washed free of adherent cells (P.C.J. Leijh, M.T. Van der Barselaar and R. Van Furth, *Infect Immun.*, 17, 313, 1977). It is then incubated with 100 μl suspension of *Escherichia coli* labelled with ^{14}C-leucine for 90 min. The residual activity in the aspirate of each well is counted, the difference between inoculated and remanant radioactivity indicating % phagocytosis (W. Steuden, Micro-organisms labelled with ^{14}C for measurement of phagocytosis, *Immunol.Ther.Exp.*, 26, 465, 1978) .Another test is macrophage migration index (K.C. Saxena, S. Puri, R. Saxena and R.P.Saxena, *Immunol.Invest.*, 20, 431, 1991). The macrophage suspension is packed in microhaematocrit capillaries (7.5 cm, 1-1.2 mm int. diameter), one end sealed and cells packed by low speed centrifugation (500xg). The capillary is cut at liquid cell interphase, placed in a migration chamber containing RPMI-1640 medium with 10% fetal calf serum and incubated overright at 37°C. The migration area can be marked and compared with untreated control to give the migration index. Additional tests to evaluate the metabolic acticity of the macrophage include incorporation of ^{14}C d-glucosamine (H. Takada, M. Tsujimoto, K. Kato, S. Kotani, S. Kusumoto, M. Inage, T. Shiba, I. Yamo, S. Kawata and K. Yokogawa, *Infect. Immun.*, 25, 48, 1979) and superoxide anion production measured by chemiluminescence.

ii. Lymphocytes: The splenic cells can be used for this purpose. They are washed and suspended in RPMI-1640 medium containing 10% fetal calf serum as for PFC assay and cultured for 3 days at 37°C in a CO_2 incubator, pulsed with H_3 thymidine and further incubated for 18 hours before harvesting and counting the incorporated radioactivity. It can be an *ex-vivo* study wherein cells from controlled and treated animals are harvested and compared or *in vitro* study in which case harvested cells can be incubated in various concentrations of test material.

3. Other Studies for Immunomodulator Acticity: More detailed studies are indicated only if initial tests indicate a potent action. These are directed to evaluate the suppressor and helper, T cell function and effect on B cell subsets. Important humoral mediators including the colony stimulating factor, Interleukin-1 and Interleukin-2 also should be assayed. Detailed procedures for these assays are available in any standard text book or laboratory manual of Immunology.

In view of the high incidence of viral hepatitis in India, CDRI programme also includes tests for antiviral activity and effect on the carrier status using the Pekin-duck model for *in vivo* studies and neutralisation of hepatitis-B viral antigens in the serum of human carriers for *in vitro* assay (R. Mehrotra, S. Rawat, D.K. Kulshreshtha , P. Goyal, G.K. Patnaik and B.N. Dhawan, *In vitro* effect of *Phyllanthus*

amarus on hepatitis B virus, *Ind.J.Med.Res.*, 93A, 71, 1991). In addition inhibition of protozoal infection of liver and spleen like Leishmaniasis is included as a test for non-specific immunostimulation, the effects being compared with MDP.

A bibliography of recent studies at CDRI with hepatoprotective plants is given in Appendix 1.

Appendix I

CDRI Publications on Hepatoprotective Activity of Natural Products (1988-1992)

1 - Hepatoprotective activity of kutkin-the iridoid glycoside mixture of *Picrorhiza kurroa*
R.A. Ansari, B.S. Aswal, R. Chander, B.N. Dhawan, N.K. Garg, N.K. Kapoor, D.K. Kulshreshtha, M. Mehdi, B.N. Mehrotra, G.K. Patnaik and S.K. Sharma.
Indian J.Med.Res., 87, 401, 1988

2 - Hepatoprotective activity of silymarin against hepatic damage in *Mastomys natalensis* infected with *Plasmodium berghei.*
R. Chander, N.K. Kapoor and B.N. Dhawan.
Indian J.Med.Res., 90, 472, 1989

3 - Strategies for scientific evaluation of Indian medicinal plants.
B.N. Dhawan
In: **Research and Development of Indigenous Drugs.**(Eds. P.C. Dandiya and S.B. Vohora) I.H.M.M.R., New Delhi, pp. 15, 1989

4 - *In-vitro* studies on the effect of certain natural products against hepatitis B virus.
R. Mehrotra, S. Rawat, D.K. Kulshreshtha, G.K. Patnaik and B.N. Dhawan
Indian J.Med.Res., 92B, 133, 1990

5 - Hepatoprotective activity of picroliv against carbon tetrachloride induced liver damage in rats.
Y. Dwivedi, R. Chander, S.K. Sharma, N.K. Kapoor, N.K. Garg and B.N. Dhawan
Indian J.Med.Res., 92, 195, 1990.

6 - Evaluation of hepatoprotective activity of picroliv in *Mastomys natalensis* infected with *Plasmodium berghei.*
R. Chander, Y. Dwivedi, R. Rastogi, S.K. Sharma, N.K. Garg and B.N. Dhawan
Indian J.Med.Res., 92, 34, 1990

7 - Safety evaluation studies on a new hepatoprotective agent, "Picroliv" in rats and monkeys.
N. Sethi, R.K. Singh, N. Sinha, G.S. Shatia, S. Srivastava and A.K. Roy.
Biol. Mem., 17, 57, 1991.

8 - Prevention of paracetamol induced hepatic damage in rats by picroliv the standardized active fraction from *Picrorhiza kurroa.*
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan
Phytother. Res., 5, 115, 1991.

9 - Absence of hepatoprotective activity in *Lagotis cashmeriana,* an adulterant to *Picrorhiza kurroa.*
B.N. Dhawan, G.K. Patnaik, D,K. Kulshreshtha and Y.K. Sarin.
Indian J. Pharmacol., 23, 121, 1991.

10- Picroliv affors protection against thioacetamide induced hepatic damage in rats.
Y. Dwivedi, R. Rastogi, S.K. Sharma, N.K. Garg and B.N. Dhawan.
Planta Med., 57, 25, 1991.

11- Choleretic effect of picroliv, the hepatoprotective principle of *Picrorhiza kurroa.*
B. Shukla, P.K.S. Visen G.K. Patnaik and B.N. Dhawan.
Planta Med., 57, 29, 1991.

12- Hepatoprotective activity of picroliv, the active principle of *Picrorhiza kurroa,* on rat hepatocytes against paracetamol toxicity.
P.K.S. Visen, B. Shukla, G.K. Patnaik, S. Kaul, N.K. Kapoor and B.N. Dhawan.
Drug. Dev. Res., 22, 209, 1991.

13- Hepatoprotective activity of picroliv isolated from *Picrorhiza kurroa* against thioacetamide toxicity in rat hepatocytes.
P.K.S. Visen, B. Shukla, G.K. Patnaik, R. Chander, V. Singh, N.K. Kapoor and B.N. Dhawan.
Phytother. Res., 5, 224, 1991.

14- Antihepatotoxic properties of picroliv, an active fraction from the rhizome of *Picrorhiza kurroa.*
R.A. Ansari, S.C. Tripathi, G.K. Patnaik and B.N. Dhawan.
J. Ethnopharmacol., 34, 61, 1991.

15- Hepatoprotective activity of picroliv against alcohol-carbon tetrachloride induced damage in rat.
S.C. Tripathi, G.K. Patnaik and B.N. Dhawan.
Indian J.Pharmacol., 23, 143, 1991.

16- Picroliv protects against monocrotaline induced hepatic damage in rats.
Y. Dwivedi, R. Rastogi, S.K. Sharma, R. Mehrotra, N.K. Garg and B.N. Dhawan.
Pharmacol. Res., 23, 399, 1991.

17- Protective activity of picroliv against damage induced by lanthanum chloride.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan.
Med. Sci. Res., 91, 711, 1991.

18- In vitro effect of *Phyllanthus amarus* on hepatitis B virus.
R. Mehrotra, S. Rawat, D.K. Kulshreshtha, P. Goyal, G.K. Patnaik and B.N. Dhawan.
Indian J.Med.Res., 93, 71, 1991.

19- Effect of picroliv on protein and nucleic acid synthesis.
V. Singh, N.K. Kapoor and B.N. Dhawan.
Indian J.Exp.Biol., 30, 68, 1992.

20- Reversal of thioacetamide induced cholestasis by picroliv.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Phytother. Res., 6, 53, 1992.

21- Effect of picroliv, a hepatoprotective agent on mice chromosomes.
A.K. Jain and N. Sethi.
Fitoterapia, 63, 255, 1992.

22- Effect of picroliv, the active principle of *Picrorhiza kurroa* on biochemical changes induced in rat liver poisoned by *Amanita phalloides*.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan.
Acta Pharmacol.Sinica, 13, 197, 1992.

23- Picroliv, Picroside-I and Kutkoside from *Picrorhiza kurroa* are scavengers of superoxide anions.
R. Chander, N.K. Kapoor and B.N. Dhawan.
Biochem. Pharmacol., 44, 180, 1992.

24- Effect of picroliv on glutathione metabolism in liver and brain of *Mastomys natalensis* infected with *Plasmodium berghei*.
R. Chandler, N.K. Kapoor and B.N. Dhawan.
Indian J.Exp.Biol., 30, 711, 1992.

25- Hepatoprotective activity in the rat of ursolic acid isolated from *Eucalytus* hybrid.
B. Shukla, P.S. Visen, G.K. Patnaik, S.C. Tripathi, R.C. Srimal, R. Dayal and P.C. Dobhal.
Phytother. Res., 6, 74, 1992.

26- Choleretic effect of andrographolide in rats and guinea pigs.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Planta Medica, 58, 146, 1992.

27- Hepatoprotective effect of the active constituents isolated from the leaves of *Ricinus communis*.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Drug Dev. Res., 26, 183, 1992.

28- Hepatoprotection by picroliv and its two iridoid glycoside components kutkoside and picroside I against galactosamine-induced damage in rat liver.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan.
Pharmacol. Toxicol., 71, 383, 1992.

29- Hepatoprotective activity of *Ricinus communis* Linn. leaves.
P.K.S. Visen, B. Shukla, G.K. Patnaik, S.C. Tripathi, D.K. Kulshreshtha, R.C. Srimal and B.N. Dhawan.
Int. J. Pharmacog., (accepted), 1992.

30- Effect of picroliv on low density lipoprotein receptor binding of rat hepatocytes in hepatic damage induced by paracetamol.
V. Singh, P.K.S. Visen, G.K. Patnaik, N.K. Kapoor and B.N. Dhawan.
Indian J.Biochem.Biophys., (accepted) 1992.

31- Reversal of galactosamine induced hepatic damage by picroliv: Study on bile flow and isolated hepatocytes (ex-vivo).
P.K.S. Visen, B. Shukla, G.K. Patnaik and B.N. Dhawan.
Planta Med., (accepted) 1992.

32- Immunostimulant activity of picroliv, the iridoid glycoside fraction of *Picrorhiza kurroa* and its protective action against *Leishmania donovani* infection in hamsters.
A. Puri, R.P. Saxena, Sumati, P.Y. Guru, D.K. Kulshreshtha, K.C. Saxena and B.N. Dhawan.
Planta Med., (accepted) 1992.

Annexure IX

A PROTOCOL FOR EVALUATING ANTI-UROLITHIATIC ACTIVITY OF PLANT PRODUCTS

B.N. DHAWAN

Central Drug Research Institute

Lucknow 226001, INDIA

Occurrence of renal and vesical stones is frequent and even endemic in certain areas of the world. The only available treatment so far was surgery till the advent of lithotripsy. These procedures, besides involving hospitalisation do not prevent recurrence, since the basic etiology has not been tackled. Many plant based remedies are used in various countries, most of them have not been scientifically evaluated either clinically or experimentally. Since no modern drugs are available, no standard protocols exist for screening and development of such products. Urolithiasis is endemic in certain parts of India and several plants are used in the Indian traditional system of medicine to treat such patients. CDRI has taken up their systematic study and developed a protocol which has been successfully used for study of *Crataeva nurvala* and *Tribulus terrestris*. The protocol being suggested for TBAM is based on the CDRI experience. The extracts should be tested for 3 types of activities:

1. Protective or curative effect on experimental urolithiasis
2. Diuretic activity
3. Antispasmodic activity on the smooth muscle of urinary tract

A. EFFECT ON EXPERIMENTAL UROLITHIASIS

I. PRODUCTION OF UROLITH

Two types of procedures are utilised for experimental production of uroliths:

1. **Foreign Body Implantation**: This technique, with minor variations, has been fallowed extensively and results in the formation of urolith around the foreign body aseptically implanted in the urinary bladder of male rat by suprapubic cystotomy under ether anaesthesia.

Initial investigators (R. McCarison, The experimental production of stone in the bladder, *Ind.J.Med.Res.*, 17, 1103, 1930) utilised pieces of human stone removed surgically and the procedure is still being used (S.K. Thind, R. Nath and R.N. Chakravarti, Experimental urolithiasis-Induction of bladder stone in rats, *Ind.J.Exp.Biol.*, 15, 30, 1977). Other foreign bodies like zinc pieces (C.W. Vermeulen, W.J. Grove, R. Goetz, H.D. Ragin and N.O. Cornell, *J.Urol.*, 64, 541, 1950) have been used and other species like the dog also utilised (C.N. Edwards, F.K. Garvey and W.H. Boyce, *J.Urol.*, 89, 207, 1963). The human stone is not always available and its composition can also vary.

CDRI has developed a procedure to overcome these problems and gets more consistent results with sterile beads of pyrex glass (15±2mg). Sample of these beads is being left at TBAM and a glass blower can easily make them. The bead is implanted aseptically in the bladder of male rats as described above and the animals sacrificed after 16 weeks. The urolith is removed for analysis along with the kidney and bladder for histopathology and blood for biochemical determinations.

2. **Production of Hyperoxaluria**: Many procedures have been employed to produce urinary hyperoxaluria, some of the oxalates get deposited into the kidney as well and thereby simulate the clinical situation. The procedures can be divided into 4 main groups.

i. Feeding of oxalic acid (3%) in the diet (C. Newcomb and S. Ranganathan, *Ind.J.Med.Res.*, 17, 1055, 1930) or its precursor like glycolic acid (F.H.C. Chow, D.W. Hamar and R.H. Udall, Prevention of oxalate and phosphate lithiasis by alanine, *Invest.Urol.*, 12, 50, 1974).

ii. Addition of certain chemicals like ethylene glycol (S.N. Gershoff and S.B. Andrus, *Proc.Soc.Exp.Biol.Med.*, 109, 99, 1962) or ethane-1-hydroxy 1,1-diphosphonate (EHDP, D. Fraser, R.G.G. Russell and O. Pohler, *Clin. Sci.*, 42, 197, 1972) to drinking water.

iii. Dietary deficiency of vitamins like B_6 (T.J. Runyan and S.N. Gershoff, The effect of vitamin B_6 deficiency in rats on the metabolism of oxalic acid precursors, *J.Biol.Chem.*, 240, 889, 1965), D or of magnessium.

iv. Other procedures like the injection of 4-hydroxy proline (2.5 g/kg i.p.)

The experience at CDRI shows that the most satisfactory procedure is giving 3% glycolic acid in diet or 1 g/kg orally daily for 40 days to male rats. Other investigators have also mixed it with the diet (S.K. Ahsan, A.H. Shah, M.O.M. Tanira, M.S. Ahmed, M. Tariq and A.M. Ageel, Studies on some herbal drugs used against kidney stones in Saudi folk medicine, *Fitoterapia*, 61, 435, 1990; P.Varalakshmi, E. Latha, Y. Shamila and S. Jayanthi, Effect of *Crataeva nurvala* on the biochemistry of the small intestinal tract of normal and stone forming rats, *J.Ethnopharmacol.*, 31, 67, 1991).

II. DRUG ADMINISTRATION

The best schedule would be to give the test drug after the stone has been fully formed but many traditional drugs are reputed to prevent the formation of stone and will, therefore, have poor curative value. Two dosage regimens have been standardised at CDRI to overcome this problem.

1. **Preventive Schedule**: In the preventive schedule the test drug is fed orally once daily for the duration of the study (16 weeks in the foreign body model and 40 days in the glycolic acid model).

2. **Curative Schedule**: In this protocol the drug administration starts only after the stone has been formed i.e. after 16 weeks in the glass bead model and for the last 10 days in the glycolic acid model.

III. DESIGN OF EXPERIMENTS

Male adult albino rats (200-225 g) are used in groups of 5 each. In the foreign body model one group contains normal animals, the second group is sham operated and in the third group foreign body is implanted but no drug is given. In view of the long duration of the experiment and the need to include 3 types of controls at least 3 drug treated group should be included, they can receive 3 test drugs or 3 doses of a single drug. The sham operated group is obviously eliminated in the experiment with glycolic acid.

IV. CRITERIA OF EVALUATION

Except for the stone analysis, which is possible in the foreign body model only, the other criteria are identical in both the type of studies. The estimations are done at the end of the period of study and mean values for each group of animals calculated. The foreign body implanted or glycolic acid treated group is compared with the normal control group while drug treated animals are compared with the urolith bearing but untreated group. Percent changes with each dose can thus be calculated.

a. Studies on the stone

1. Radiological study: This is done before sacrificing the animals in the experiments with rats having implanted glass bead. An anterio-postesior radiograph of the pelvic region is obtained in each animal for comparison of the size of urolith.

2. Weight: The stone is weighed on extraction and dried to a constant weight to determine the total weight of the urolith formed and also the moisture content.

3. Chemical Analysis: The following parameters are estimated:

i. *Ash Content* by heating the moisture free sample at 600°C for 16 hours in an open platinum crucible. The ash is then dissolved in 6 ml of 0.7 N HCl, diluted with distilled water to 25 ml and used for the determination of calcium, magnessium and phosphates.

ii. *Calcium* can be determined by the Clark Collip calcium oxalate precipitation method (R.B.H. Gradwohi, **Clinical Laboratory Methods and Diagnosis**, Vol.1, C.V. Mosby and Co., 5th Ed., p. 442, 1956) taking 1 ml of the solution and using Gilmont's microbiurettee for titration. Alternatively the procedure of B.C. Ray Sarkar and V.P.S. Chauhan (*Anal.Biochem.*, 20, 155, 1967) can be used.

iii. *Phosphates* are also determined in 1 ml of ash solution using the method of Fiske and Subbarow (C.H. Fiske and Y. Subbarow, The colorimetric determination of phosphorus, *J.Biol.Chem.*, 66, 375, 1925).

iv. *Magnessium* estimation in the solution is done by the Titan yellow colorimetric method (M. Orange and C. Rhein, Microestimation of magnessium in body fluids, *J.Biol.Chem.*, 189, 379, 1951).

v. *Oxalate* has to be determined in the urolith without ashing by titration with N/10 $KMnO_4$ at 90°C in a Gilmont's microbiurettee or by colorimetry (A. Hodgkinson and A. Williams, An improved colorimetric procedure for urine oxalate, *Clin.Chim.Acta.*, 36, 127, 1972).

vi. *Cholesterol* also should be determined in the urolith and can be done with the procedure used for its estimation in the serum (A. Zlatkis, B. Zak and A.J. Boyle, A new method for direct determination of serum cholesterol, *J.Lab.Clin.Med.*, 41, 486, 1953).

b. **Urine Examination**

The animals are put in individual metabolic cages and 24 hr urine collected a day before the sacrifice. Its specific gravity and pH is determined and the sample is divided in 3 parts for other estimations.

1. Qualitative examination: Routinely available diagnostic strips (eg. Ames Division, Miles Laboratories) can be used to detect the presence of glucose, ketone, bilirubin, blood, urobilinogen and proteins. The strips provide semi-quantitative data. All these substances are not present normally in the urine.

2. Microscopic Examination: The urine sample is centrifuged at low speed and the deposit is examined microscopically for crystals, casts, RBC and other cellular constituents (J.A. Kolmer, H.S. Earle and W.R. Howard, **Approved Laboratory Techniques**, Appleton Century Crofts Inc. New York, 5th Ed., p. 173, 1951). Normal urine may only show some triple phosphate or urate crystals and epithelial cells.

3. Chemical Estimation: The urine samples are analysed for creatinine (J.C. Todd and A.H. Sanford, 1943, *In*: **Clinical Diagnosis by Laboratory Methods**, W.B. Saunders Co., Philadelphia, 10th Ed., p.365), calcium (Ray Sarkar and Chauhan, cited above or J. Hooper, Microdetermination of urine calcium, *J.Med.Lab.Technol.*, 13, 470, 1956), phosphates (Fiske and Subbarow, cited above) and oxalate (Hodgkinson and Williams, cited above) content.

The following values have been obtained for the urine of normal rats at CDRI

Specific gravity	1.01
pH	6.5
Creatinine	7.1±8.2 mg/dL
Calcium	7.3±0.8 mg/dL
Phosphate	45.3±4.5 mg/dL Phosphorus
Oxalate	4.2±0.5 mg/dL

c. **Blood Examination**

Blood should be collected from the retero-orbital plexus at the time of sacrifice, 0.5 ml in a heparinied vial and 5 ml for separation of serum.

1. Total Leucocyte Count is performed by the standard procedure in a counting chamber under the microscope or using an automated counter like the Coulter counter. The normal count varies between 7.5-14x10³/cumm.

2. Chemical Constituents: Serum creatinine, calcium and phosphates are determined as described for the urine. Blood urea can also be determined by the method of Todd and Stanford (cited above) or by the urease method (E.P. Marbach, Clin. Chem., 8, 130, 1962). The normal levels are creatinine (8-10 mg/L), calcium (2.3-2.8 μMol/L), phosphates (1.36-2.97 μMol/L) and urea (4.32-12-95 uMol/L).

3. **Histophatology**

Pieces of kidney and urinary bladder are fixed in 10% neutral formalin. After embedding in paraffin was 5 μ sections are cut serially and stained with haematoxylin and eosin for microscopic examination. Weight of the kidney is also recorded.

V. **EFFECTS OF GLASS BEAD IMPLANTATION**

1. **Stone:** The average weight of the formed urolith around the glass bead is 19±2 mg and analysis reveals (mg per 100 mg stone powder), moisture (0.03), ash (0.21), oxalate (0.06), calcium (16.54), phosphorus (8.95), magnesium (0.3) and cholesterol (0.07). It is clearly visualised radiologically.

2. **Urine:** Qualitative examination shows traces of blood. Ocassionally proteins, ketone or bilurubin may be seen. Microscopic examination reveals RBC, pus cells; crystals of triple phosphate, calcium phosphate and uric acid and moderate numbers of hyaline and blood casts. Among the chemical constituents, there is a significant increase in the concentration of calcium, phosphates and oxalate while creatinine is unaffected.

3. **Blood:** The development of urolith is associated with significant leucocytosis. The blood urea may also show a significant increase but the levels of other constituents like calcium, phosphorus and creatinine remain unchanged.

4. **Histophathological Changes:** There are generally very few changes in the kidney, in some animals interstitial patchy inflammatory changes may be observed with dilation of the tubules. Similar changes have been observed by Thind et al (1977, cited above). There are, however, marked changes in the mucosa of the urinary bladder. There is considerable thickening and folding of the mucosa and inflammatory cells appear in submucosal layers along with edema and often engorged capillaries.

Treatment with an effective agent should be able to prevent most of these changes in a dose related manner and less effectively to reverse them when given to animals where urolith has already been formed. Some of these effects with Crataeva nurvala have been reported in the MSS from CDRI attached at Appendix 1.

VI. EFFECTS OF GLYCOLIC ACID

1. **Urine:** There is a marked increase in the concentration of calcium, oxalate and phosphates. The calcium oxalate excretion is increased up to 30-fold. The magnessium levels drop leading to a reversal of Ca/Mg ratio.

2. **Blood:** There are no significant changes in the blood chemistry and leucocyte count.

3. **Kidney:** The weight of the kidney increases (both wet and dry) leading to upto a two-fold increase in kidney/body weight ration. The levels of calcium, oxalate and phosphates are raised and white patches may appear on the kidney due to deposition of calcium oxalate.

The changes can be prevented in a concentration dependant manner by plants extracts having antiurolithiatic activity (Ahsan et al, cited above; P.Varalakshmi, Y. Shamila and E. Latha, Effect of Crataeva nurvala in experimental urolithiasis, J.Ethnopharmacol. 28, 313, 1990).

B. DIURETIC ACTIVITY

The diuretic activity is tested in overnight fasted adult male rats in groups of 5 each. They are hydrated with 5 ml/100 g normal saline orally (A.De Felice, A.Harris and A.Brousseau, Use of hydrated rats to assay diuretic and antidiuretic activity of drugs, Drug.Dev.Res.,22,95,1990) and kept in

metabolic cages so that urine can be collected for the next 6 hours. The control group receive only saline, for comparison 70 mg/kg urea (D.S. Bhakuni, M.L. Dhar, M.M. Dhar, B.N. Dhawan and B.N. Mehrotra,Screening of Indian plants for biological activity, Part II, Ind.J.Exp.Biol., 7, 250,1969) or mg/kg chlorthiazide is given to a second group of animals while the remaining groups are given the test compounds. The volume of urine, its osmolality (with a micro osmometer) and concentration of K+ and Na+(Flame photometer) are measured to get the activity index and activity profile of the test drug. The initial dose should be 1/4th LD_{50}.

C. ANTISPASMODIC ACTIVITY

Some plant products are reported to help in expulsion of the stone. If the stone is in the kidney, the descent will be helped by relaxation of the ureter. Stones which can travel down the ureter will usually pass through the urethra. The bladder stones, on the other hand, will need contraction of the bladder and relaxation of urethra for their expulsion. It must be pointed out that only small stones can be passed out in this may either from the kidney or from the bladder. The effect of the extracts should be studied on all these smooth muscles and these experiments can be performed in vitrol.

1.**Ureter**: We have used the ureter of cat (G.K. Patnaik, K.K.Banaudha, K.A.Khan, A.Shoeb and B.N.Dhawan, Spasmolytic activity of angelicin: a coumarin from Heracluem thomsoni. Planta Med., 53, 517, 1987). A 2-3 cm long piece is suspended in aerated Tyrode solution (pH7.4) at 35°C and contraction can be induced by KCI (100 µg/ml), which should be antagonised by a spasmolytic agent. A similar preparation from other species like the rat or the guinea pig should work equally well.

2. **Urinary bladder and urethra**: The bladder and urethra from male mice is isolated and muscle strips are prepared (M.Kimura,K.Nakase,T.Sonobe and N.Mori, Micturition effect of pollen extract: contractile effects on bladder smooth muscle and inhibitory effect on urethral smooth muscle of mouse and pig, Planta Med.,52,285, 1986). The strips are suspended in a modified Kreb's solution (NaCl(122),KCI(5.9),$CaCl_2$(1.2), $NaHCO_3$(15.5) and glucose (11.5) µ M) at 37°C. The resting tension is adjusted to 150 mg for the bladder and 100 mg for urethra and contraction recorded with an isometric transducer on a polygraph.

The bladder strip contracts in response to PG $F_{2\alpha}$ (3 µg/ml) and the agonist for urethra is noradrenaline (0.3 ug/ml); the bladder muscle may show spontaneous activity also. Similar preparations can be made from other species of animals as well.

APPENDIX - 1

Antiurolithiatic activity of Crateva nurvala Buch. Ham. alcoholic extract on rats *

R. Anand, G.K. Patnaik, D.K. Kulshreshtha, B.N. Mehrotra, R.C. Srimal and B.N. Dhawan **

ICMR Centre for Advanced Pharmacological Research on Tradition Remedies, Central Drug Research Institute, Lucknow 226001

---

* CDRI Communication No. : 4995

** Address for correspondence : Prof. B.N. Dhawan, Director
Central Drug Research Institute
Lucknow 226001
India

SUMMARY

The alcoholic extract of the stem bark of the Indian medicinal plant Crateva nurvala showed a significant dose dependent (25-100 mg/kg, p.o.) prophylactic activity against experimentally induced urolith formation in rats. It also reversed the biochemical parameters in urine, blood and serum, and brought back histophathological changes towards normal.

INTRODUCTION

In the modern system of medicine obstructive uropathy is still a challenging problem. The available operative and lithotripsy procedures are not free from complications and recurrence of stone formation is quite common. On the other hand, in the traditional Indian system of medicine, several plants are claimed to be used successfully in the treatment of urolithiasis[1].

The medicinal value of Crateva nurvala (Hindi: Varuna) belonging to Capparidaceae family, has been described against a wide variety of urinary disorders, including urolithiasis, in the ancient Ayurvedic text " Sushruta Samhita"[2]. C. nurvala stem bark also forms a major constituent of many Ayurvedic preparations currently used for treatment of urolithiasis.

The present investigation deals with an evaluation of the possible antiurolithiatic property of ethanolic extract of C. nurvala stem bark in rats against artificially induced urolithiasis.

EXPERIMENTAL

Plant material

The botanically identified plant material was collected in the month of September from Saneh fores, Pauri Garhwal, U.P. A voucher specimen has been preserved in the herbarium of the Institute. The air dried stem bark powder was exhaustively percolated with 95% ethanol and concentrated under reduced pressure below 50° C. The resultant dry ethanolic extract (yield 3%) of C. nurvala was used for experimental studies.

Animals

Male adult albino rats of Charles Foster strain (225-250g) obtained from C.D.R.I. animal facility were used. They were housed under standard animal husbandry conditions and had free access to pellet diet and tap water during the study. Urolithiasis was induced by the foreign body implantation technique as described by Thind et al[3]. In the ether anaesthetised rats, a pre weighed sterile glass bead (15+2mg) was implanted in the lumen of urinary bladder. The bladder was closed with a single stitch and the abdomen was closed in layers. Rats were allowed to recover from the anaesthesia.

Dose schedule:

In the traditional system of medicine the daily dose of crude plant powder is 10 g orally for human being (approx. 60 kg). The equivalent rat dose is about 7 times higher than man[4]. As the yield of the alcoholic extract is 3%, we had selected 25 to 100 mg/kg of the alcoholic extract for studying the dose depend effect.

The animals were divided into the following six groups containing five animals each:

Group I - Normal untreated and unoperated control

Group II - Sham operated

Group III - Control (only foreign body implanted)

Group IV - Foreign body implanted and treatment with the plant extract (25 mg/kg,p.o.) for 16 weeks from the day of implantation.

Group V - Group IV schedule with 50 mg/kg p.o. plant extract

Group VI - Group IV schedule with 100 mg/kg p.o. plant extract

The animals were observed for 16 weeks and were than sacrificed by decapitation after X-ray examination.

1. Examination of stone

(i) Radiographical studies

Radiographical examination was done before sacrificing the animal to confirm the formation of stone. The animal was kept under light ether anaesthesia in anteroposterior position to expose the pelvic region, on a PLECDOR 3 X-ray machine (Siemens India Ltd.). Film was kept 100 cm away from the tube.

(ii) Weight of stone

The difference between the weight of the implanted glass bead at the time of implantation and final weight of the dried calculi taken out from the bladder at the end of 16 week period indicated the amount of deposited stone.

(iii) Chemical analysis

Stones obtained from the bladder were pooled according to the respective groups and analysed by standard techniques[5] for moisture, ash, oxalate, calcium, phosphorus, magnesium and cholesterol content.

2. Urine examination

Twenty four hour urine samples were collected by putting the animals individually in metabolic cages before sacrifice.

(i) Microscopic examination

Urine samples were centrifuged at low speed and the deposits were examined microscopically for sediments, crystals, casts and cells[6].

(ii) Biochemical tests

Various parameters were analysed using diagnostic reagent strip (Ames Division, Miles India Ltd., Baroda). These included specific gravity, pH and presence of glucose, ketone, bilirubin, blood, urobilinogen and proteins.

(iii) Electrolytes

Urine samples were analysed for calcium[7] and phosphorus[8].

3. Total blood leucocyte count

This was done with the help of a Coulter Counter (Model ZF) using 0.5 ml of heparinised blood collected from orbital plexus.

4. Serum Examination

Blood (5ml) was collected from orbital plexus and centrifuged at 2000 rpm for 20 minutes in REMI cooling centrifuge, C-23. The serum was separated for estimation of urea[9], creatinine[9], calcium[7] and inorganic phosphorus[8].

5. Histopathology

Tissues of the kidney and urinary bladder were fixed in 10% neutral formaline and paraffin blocks were made. These blocks were sectioned (50) and stained with hematoxylin and eosin for microscopic examination.

6. Statistical analysis

Results shown in the tables have been reported as mean +SD. Each parameters was analysed by one way analysis of variance considering the homogeneity of variance. Multiple comparisons among different group means were done by Newman-Keul's Test.

RESULTS

Various parameters of the sham operated group did not vary from those of the untreated and unoperated normal animals.

(1) Examination of stone

(i) Radiographical studies

Urolith formation was confirmed by X-ray examination. A dose dependent reduction in the size of the stones was observed with 25, 50 and 100 mg/kg doses. The 100 mg/kg treated group showed very little deposition of stone in comparison to the untreated control group.

(ii) Weight of stone

The ethanolic extract of C.nurvala (100 mg/kg p.o.) showed 81.6% reduction in the stone deposition as compared to the control group. With 25 and 50 mg/kg doses the reduction was found to be 18.3 and 42.3% respectively (Table 1).

(iii) Chemical analysis of stones

All the observed parameters, except moisture content, were found to be reduced in a dose dependent manner in the drug treated animals. Magnesium level was, however, found unchanged. The results are summarized in Table 2.

(2) Urine examination

(i) Microscopic examination

R.B.C. pus cells, crystals and casts were found in the urine of control (Group III) animals in significant numbers. Marked dose dependent reduction in all the parameters was found in the urine samples of animals treated with different doses of ethanolic extract of C.nurvala (Table 3).

(ii) Biochemical tests

In the plant extract treated groups specific gravity and pH of urine were found within normal range. Protein, glucose, ketone, bilirubin and urobilinogen were either absent or found in small quantity comparable to normal urine, except blood, which was found in traces in one treated animal.

(iii) Electrolytes

The levels of calcium and inorganic phosphorus were found to be increased in the urine of control group. In the extract treated groups the levels showed a dose dependent reduction towards normalization (Table 4).

(3) Total leucocyte count

The count was raised in the control group whereas a dose dependent restoration was found in the treated groups (Table 5).

(4) Serum analysis

The levels of calcium (ranging from 9.01 to 10.79 mg/dl), inorganic phosphorus (ranging from 7.27 to 8.70 mg/dl) and creatinine (0.64-0.71 mg/dl) in the serum of all the groups remained unaffected. The altered urea level in stone bearing animals was significantly reverted towards normal by plant extract treatment.

(5) Histopathology

(i) Urinary bladder

The urinary bladder of the control group showed thickening/folding as signs of inflammatory changes. The histopathological findings of the bladder of the C. nurvala treated animals indicated a dose dependent reduction in the severity of these changes. In the 100 mg/kg extract treated group there was almost complete normalization.

(ii) Kidney

This histological findings indicated normal tissue in all the groups.

DISCUSSION

It is well known that the foreign body acts as nidus around which crystals tend to precipitate leading to formation of stone in the urinary

bladder. Crystal growth in vivo consists of deposition from a saturated or super saturated solution, or precipitation from chemical reaction, followed by the deposition on the nucleus[9]. These facts are supported by increased levels of Ca^{2+} and PO^{3-} ions in the urine of stone forming rats as these ions tend to precipitate at alkaline pH. A similar mechanism would presumably be operative in the animals of the present study leading to formation of vesical calculus.

The increased level of Ca ion in the urolith in the control (glass bead implanted) group in our study is in agreement with other reports[11] suggesting the pattern of initial deposition of Ca^{2+} and PO^{3-} ions in the form of a mineral phase bound to the matrix and the subsequent growth of the preformed mineral phase by the deposition of the above ions.

The ethanolic extract of stem bark of C.nurvala significantly inhibited the formation of urolith in a dose dependent manner and also reversed the various biochemical parameters towards their normal levels, that are known to precipitate the urolith formation.

It is not possible to postulate the precise mechanism of action of the extract on the basis of available data. It may be inhibiting the mineral phase formation and its subsequent growth. The other possible mechanism may be by stimulation of demineralisation or dissolution of the mineral phase.

The presence of triple phosphates in the urine, increased blood leucocyte count and appearance of R.B.C. in urine of the control bead implanted animals indicate inflammation and urinary infection, possibly with urea splitting organisms which are known to increase the alkalinity of the urine. These factors would increase the rate of precipitation of calcium phosphate and magnesium ammonium phosphate[12]. A decrease of these substances

as well as of oxalate and cholesterol in the calculus suggests that C.nurvala prevents their deposition around the nidus.

The present investigation showed that the plant extract markedly prevents the formation of urolith. This confirms the utility of use of the plant in Ayurvedic system of medicine against urolithiasis. Further study is in progress for identification and detailed preclinical evaluation of the active constituent of the plant.

References

1. Mukerjee T., Bhalla N., Aulakh G.S., Jain H.C.
Indian Drugs. 21 224 (1984)

2. Bhisha gratna K.L.
"English translation of Sushruta Samhita"
Chowkhamba Sanskrit Series Office, Varanasi, (1981), pp 7-12.

3. Thind S.K., Nath R., Chakravarti R.N.
Indian J. Exp. Biol. 15, 30 (1977)

4. Freireich E.J.
Cancer chemother. Repts. 50, 219 (1966)

5. Thind S.K., Nath R.
Indian J. Med. Res. 57, 1790 (1969)

6. Kolmer J.A., Earle H.S., Howard W.R.
"Approved laboratory technic"
Appleton Century Crofts Inc., New York, (1951), pp. 173-189

7. Ray Sarkar B.C., Chauhan U.P.S.
Anal. Biochem. 20, 155 (1967)

8. Fiske C.H., Subbarow Y.
J. Biol. Chem. 66, 375 (1925)

9. Davidsohn I., Henry J.B.
"Todd-Sanford clinical diagnosis by laboratory methods"
W.B. Saunders Company, Philadelphia, (1969), pp. 365-370

10. Lonsdale K.
Science. 159, 1199 (1968)

11. Jethi R.K., Duggal B., Sahota R.S., Gupta M., Sofat I.B.
Indian J. Med. Res. 78, 422 (1983)

12. McGeown M.G., Bull G.M.
Brit. Med. Bull. 13, 53 (1957)

Table 1 Effect of ethanolic extract of C.nurvala (CN) on the weight of the bladder stones.

Treatment mg/kg os	Increase in the wt. of stone (mg)	% Reduction
Control	19.04±2.04	-
CN 25	15.56±2.25*	18.28
CN 50	10.98±1.74**	42.33
CN 100	3.51±0.25**	81.56

Values are Mean ±SD. n=5 per group,
*p<0.05, ** p<0.01 Vs the control group.

Table 2 Chemical analysis of stones from control and C.nurvala treated groups.

Parameters	Controls	C.nurvala(mg/kg os)		
		25	50	100
Moisture	0.032	0.093	0.098	0.143
Ash	0.212	0.198	0.162	0.110
Oxalate	0.063	0.061	0.053	0.056
Calcium	16.542	14.367	14.039	12.401
Phosphorus	8.950	7.639	7. 325	5.871
Magnesium	0.300	0.378	0.351	0.433
Cholesterol	0.071	0.069	0.068	0.068

Stones obtained from animals of each group (n=5) were pooled together and powdered for analysis. Values are expressed in mg per 100 mg stone powder.

Table 3: Microscopic examination of urine of normal, sham operated, control and C.nurvala treated animals.

Parameters	Normal	Sham Operated	Controls	C.nurvala (mg/kg os) 25	50	100
1. R.B.C.	-	-	+++	+	+	+
2.Pus Cells	-	-	++	+	-	-
3. Casts						
Blood	-	-	+	-	-	-
Hyaline	-	-	+	+	-	-
4. Crystals						
Triple Phosphate	-	-	++	+	-	-
Calcium Phosphate	-	-	+++	++	+	-
Uric Acid	+	+	+++	++	+	+

Absent -, Moderate ++
Low +, High +++

Table 4: Urinary electrolytes in normal, sham operated, control and treated animals.

Groups	Calcium (mg/dl)	Phosphorus (mg/dl)
Normal	8.64±2.15	50.62±6.56
Sham operated	8.41±1.54	49.71±6.14
Control	11.01±1.72*	62.22±4.27*
C.nurvala (mg/kg os)		
25	10.25±2.31	60.31±6.21
50	8.82±1.16*	55.02±4.02*
100	8.00±1.02*	50.73±5.22*

Values are mean ±SD, n=5 per group.
Normal group compared with sham operated group.
Control group compared with Sham operated group.
Treated groups compared with control group.
* $p<0.05$

Table 5: Total blood leucocyte count and serum urea levels of normal, sham operated, control and C.nurvala treated animals.

Groups	Leucocytes (10^9/l)	Urea (mg/dl)
Normal	15.00±2.54	39.97±3.46
Sham operated	15.25±3.18	38.56±4.34
Control	21.45±2.55**	45.13±3.59*
C.nurvala (mg/kg os)		
25	18.69±3.43	44.19±4.85
50	16.88±2.56*	40.05±2.65*
100	15.55±1.35**	38.0±2.46*

Values are mean ±SD, n-5 per group.
Normal group compared with Sham operated group.
Control group compared with Sham operated group.
Treated groups compared with control group.
*$p<0.05$, ** $p<0.01$

Annexure X

LIST OF REPRINTS

A - Hepatoprotective Agents

1 - Hepatoprotective activity of kutkin-the iridoid glycoside mixture of *Picrorhiza kurroa*
R.A. Ansari, B.S. Aswal, R. Chander, B.N. Dhawan, N.K. Garg, N.K. Kapoor, D.K. Kulshreshtha, M. Mehdi, B.N. Mehrotra, G.K. Patnaik and S.K. Sharma.
Indian J.Med.Res., 87, 401, 1988

2 - Hepatoprotective activity of silymarin against hepatic damage in *Mastomys natalensis* infected with *Plasmodium berghei*.
R. Chander, N.K. Kapoor and B.N. Dhawan.
Indian J.Med.Res., 90, 472, 1989

3 - *In-vitro* studies on the effect of certain natural products against hepatitis B virus.
R. Mehrotra, S. Rawat, D.K. Kulshreshtha, G.K. Patnaik and B.N. Dhawan
Indian J.Med.Res., 92B, 133, 1990

4 - Hepatoprotective activity of picroliv against carbon tetrachloride induced liver damage in rats.
Y. Dwivedi, R. Chander, S.K. Sharma, N.K. Kapoor, N.K. Garg and B.N. Dhawan
Indian J.Med.Res., 92, 195, 1990.

5 - Evaluation of hepatoprotective activity of picroliv in *Mastomys natalensis* infected with *Plasmodium berghei*.
R. Chander, Y. Dwivedi, R. Rastogi, S.K. Sharma, N.K. Garg and B.N. Dhawan
Indian J.Med.Res., 92, 34, 1990

6 - Prevention of paracetamol induced hepatic damage in rats by picroliv the standardized active fraction from *Picrorhiza kurroa*.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan
Phytother. Res., 5, 115, 1991.

7 - Absence of hepatoprotective activity in *Lagotis cashmeriana*, an adulterant to *Picrorhiza kurroa*.
B.N. Dhawan, G.K. Patnaik, D.K. Kulshreshtha and Y.K. Sarin.
Indian J. Pharmacol., 23, 121, 1991.

8 - Picroliv affors protection against thioacetamide induced hepatic damage in rats.
Y. Dwivedi, R. Rastogi, S.K. Sharma, N.K. Garg and B.N. Dhawan.
Planta Med., 57, 25, 1991.

9 - Choleretic effect of picroliv, the hepatoprotective principle of *Picrorhiza kurroa*.
B. Shukla, P.K.S. Visen G.K. Patnaik and B.N. Dhawan.
Planta Med., 57, 29, 1991.

10- Hepatoprotective activity of picroliv. the active principle of *Picrorhiza kurroa*, on rat hepatocytes against paracetamol toxicity.
P.K.S. Visen, B. Shukla, G.K. Patnaik, S. Kaul, N.K. Kapoor and B.N. Dhawan.
Drug. Dev. Res., 22, 209, 1991.

11- Hepatoprotective activity of picroliv isolated from *Picrorhiza kurroa* against thioacetamide toxicity in rat hepatocytes.
P.K.S. Visen, B. Shukla, G.K. Patnaik, R. Chander, V. Singh, N.K. Kapoor and B.N. Dhawan.
Phytother. Res., 5, 224, 1991.

12- Antihepatotoxic properties of picroliv, an active fraction from the rhizome of *Picrorhiza kurroa*.
R.A. Ansari, S.C. Tripathi, G.K. Patnaik and B.N. Dhawan.
J. Ethnopharmacol., 34, 61, 1991.

13- Hepatoprotective activity of picroliv against alcohol-carbon tetrachloride induced damage in rat.
S.C. Tripathi, G.K. Patnaik and B.N. Dhawan.
Indian J.Pharmacol., 23, 143, 1991.

14- Picroliv protects against monocrotaline induced hepatic damage in rats.
Y. Dwivedi, R. Rastogi, S.K. Sharma R. Mehrotra, N.K. Garg and B.N. Dhawan.
Pharmacol. Res., 23, 399, 1991.

15- Protective activity of picroliv against damage induced by lanthanum chloride.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan.
Med. Sci. Res., 91, 711, 1991.

16- In vitro effect of *Phyllanthus amarus* on hepatitis B virus.
R. Mehrotra, S. Rawat, D.K. Kulshreshtha, P. Goyal, G.K. Patnaik and B.N. Dhawan.
Indian J.Med.Res., 93, 71, 1991.

17- Effect of picroliv on protein and nucleic acid synthesis.
V. Singh, N.K. Kapoor and B.N. Dhawan.
Indian J.Exp.Biol., 30, 68, 1992.

18- Reversal of thioacetamide induced cholestasis by picroliv.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Phytother. Res., 6, 53, 1992.

19- Effect of picroliv, a hepatoprotective agent on mice chromosomes.
A.K. Jain and N. Sethi.
Fitoterapia, 63, 255, 1992.

20- Effect of picroliv, the active principle of *Picrorhiza kurroa* on biochemical changes induced in rat liver poisoned by *Amanita phalloides*.
Y. Dwivedi, R. Rastogi, N.K. Garg and B.N. Dhawan.
Acta Pharmacol.Sinica, 13, 197, 1992.

21- Picroliv, Picroside-I and Kutkoside from *Picrorhiza kurroa* are scavengers of superoxide anions.
R. Chander, N.K. Kapoor and B.N. Dhawan.
Biochem. Pharmacol., 44, 180, 1992.

22- Effect of picroliv on glutathione metabolism in liver and brain of *Mastomys natalensis* infected with *Plasmodium berghei*.
R. Chandler, N.K. Kapoor and B.N. Dhawan.
Indian J.Exp.Biol., 30, 711, 1992.

23- Choleretic effect of andrographolide in rats and guinea pigs.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Planta Medica, 58, 146, 1992.

24- Hepatoprotective effect of the active constituents isolated from the leaves of *Ricinus communis*.
B. Shukla, P.K.S. Visen, G.K. Patnaik and B.N. Dhawan.
Drug Dev. Res., 26, 183, 1992.

B - Curcumin

25- Pharmacology of diferuloyl methane (curcumin) a non-steroidal anti-inflammatory agent.
R.C. Srimal and B.N. Dhawan
J. Pharm.Pharmacol., 25, 447, 1973

26- Preliminary study on antirheumatic activity of curcumin (diferuloyl methane)
S.D. Deodhar, R. Sethi and R.C. Srimal
Ind.J.Med.Res., 71, 632, 1980

27- Modification of certain inflammation-induced biochemical changes by curcumin.
R. Srivastava and R.C. Srimal
Ind.J.Med.Res., 81, 215, 1985

28- Anti-thrombotic effect of curcumin.
R. Srivastava, M. Dikshit, R.C. Srimal and B.N. Dhawan.
Thromb.Res., 40, 413, 1985

29- Effect of curcumin on platelet aggregation and vascular prostacyclin synthesis.
R. Srivastava, V. Puri, R.C. Srimal and B.N. Dhawan
Arzneim.Forsch., 36, 715, 1986

30- Curcumin.
R.C. Srimal
Drugs of Future, 12, 331. 1987

31- Inhibition of neutrophil response by curcumin.
R. Srivastava
Agents Actions, 28, 298, 1989

32- Curcumin: A potent inhibition of leukotriene B_4 formation in rat peritoneal polymorphonuclear neutrophils (PMNL).
H.P.T. Ammon, M.I. Anazodo, H.Safayhi, B.N. Dhawan and R.C. Srimal.
Planta Med., 58, 226, 1992

C - Other Natural Products

33- Evaluation of spasmolytic activity of Clausmarin-A. A novel coumarin from *Clausena pentaphylla* (Roxb.) D.C.
G.K. Patnaik, and B.N. Dhawan
J.Ethnopharmacol., 16, 127, 1982

34- Spasmolytic activity of Angelicin: A coumarin from *Heracleum thomsoni (Linn.)*
G.K. Patnaik, K.K. Banaudha, K.A. Khan, A. Shoeb and B.N.Dhawan
Planta Med., 53, 517, 1987

35- Adaptogenic activity of Indian *Panax pseudoginseng*
P.R. Dua, G. Shankar, R.C. Srimal, K.C. Saxena, R.P. Saxena, A. Puri, B.N. Dhawan, Y.N. Shukla, R.S. Thakur and A. Husain.
Ind.J.Exp.Biol., 27, 631, 1989.

D - Reviews

36- Research on plants for fertility regulation in India.
V.P. Kamboj and B.N. Dhawan.
J.Ethnopharmacol., 6, 191, 1982.

37- Medicinal Plants.
R.P. Rastogi, B.N. Dhawan and M.M. Dhar.
40 Years of Research - A CSIR Overview, Council of Scientific and Industrial Research, New Delhi, p.329. 1987.

38- Strategies for scientific evaluation of Indian medicinal plants.
B.N. Dhawan.
Research and Development of Indigenous Drugs, Eds., P.C. Dandiya and S.B. Vohora, IHMMR, New Delhi, p.15, 1989.

39- Anticancer and antiviral activities in Indian medicinal plants: A review.
R.P. Rastogi and B.N. Dhawan.
Drug Develop. Res., 19, 1, 1990.

40- Methods for biological assessment of plant medicine.
B.N. Dhawan.
The Medicinal Plant Industry, Ed., R.O.B. Wijesekera, CRC Press, Boca Raton, U.S.A. p.77, 1991.

41- Recent developments from Indian medicinal plants.
B.N. Dhawan and R.P. Rastogi.
The Medicinal Plant Industry, Ed., R.O.B. Wijesekera, CRC Press, Boca Raton, U.S.A. p.185, 1991.

42- In pursuit of new drugs - Central Drug Research Institute.
B.N. Dhawan and Z. Mani.
Curr.Sci., 62, 396, 1992.

E - Biological Screening

43- Screening of Indian plants for biological activity, Part I.
M.L. Dhar, M.M. Dhar, B.N. Dhawan, B.N. Mehrotra and C. Ray
Ind.J.Exp.Biol., 6, 233, 1968.

44- Screening of Indian plants for biological activity, Part II.
D.S. Bhakuni, M.L. Dhar, M.M. Dhar, B.N. Dhawan and B.N. Mehrotra
Ind.J.Exp.Biol., 7, 250, 1969.

45- Screening of Indian plants for biological activity, Part III.
D.S. Bhakuni, M.L. Dhar, M.M. Dhar, B.N. Dhawan, B. Gupta and R.C. Srimal
Ind.J.Exp.Biol., 9, 91, 1971.

46- Screening of Indian plants for biological activity, Part IV.
M.L. Dhar, M.M. Dhar, B.N. Dhawan, B.N. Mehrotra, R.C. Srimal and J.S. Tandon
Ind.J.Exp.Biol., 11, 43, 1973.

47- Screening of Indian plants for biological activity, Part V.
M.L. Dhar, B.N. Dhawan, C.R. Prasod, R.P. Rastogi, K.K. Singh and J.S. Tandon B.N.
Ind.J.Exp.Biol., 12, 512, 1974.

48- Screening of Indian plants for biological activity, Part VI.
B.N. Dhawan, G.K. Patnaik, R.P. Rastogi, K.K. Singh and J.S. Tandon
Ind.J.Exp.Biol., 15, 208, 1975.

49- Screening of Indian plants for biological activity, Part VII. Spermicidal activity of Indian plants.
B.S. Setty, V.P. Kamboj and N.M. Khanna
Ind.J.Exp.Biol., 15, 231, 1977.

50- Screening of Indian plants for biological activity, Part VIII.
C.K. Atal, J.B. Srivastava, B.K. Wali, R.B. Chakravarty, B.N. Dhawan and R.P. Rastogi.
Ind.J.Exp.Biol., 15, 208, 1978.

51- Screening of Indian plants for biological activity, Part IX.
B.N. Dhawan, M.P. Dubey, B.N. Mehrotra, R.P. Rastogi and J.S. Tandon
Ind.J.Exp.Biol., 18, 594, 1980.

52- Screening of Indian plants for biological activity, Part X.
B.S. Aswal, D.S. Bhakuni, A.K. Goel, K.Kar, B.N. Mehrotra and K.C. Kukherjoc.
Ind.J.Exp.Biol., 22, 312, 1984.

53- Screening of Indian plants for biological activity, Part XI.
B.S. Aswal, D.S. Bhakuni, A.K. Goel, K. Kar, B.N. Mehrotra and G.K. Patnaik
Ind.J.Exp.Biol., 22, 487, 1984.

54- Screening of Indian plants for biological activity, Part XII.
Z. Abraham, D.S. Bhakuni, H.S. Garg, A.K. Goel, B.N. Mehrotra and G.K. Patnaik
Ind.J.Exp.Biol., 24, 483, 1986.

55- Screening of Indian plants for biological activity, Part XIII.
D.S. Bhakuni, A.K. Goel, S. Jain, B.N. Mehrotra, G.K. Patnaik and V. Prakash
Ind.J.Exp.Biol., 26, 883, 1986.

56- Screening of Indian plants for biological activity, Part XIV.
D.S. Bhakuni, A.K. Goel, A.K. Goel, S. Jain, B.N. Mehrotra and R.C. Srimal
Ind.J.Exp.Biol., 28, 619, 1990.

F - Other Reprints

57- Chemical analysis of urinary calculi in Chandigarh area.
S.K. Thind and R. Nath
Ind.J.Med.Res., 57, 1790, 1969.

58- Experimental urolithiasis - Induction of bladder stones in rats.
S.K. Thind, R. Nath and R.N. Chakravarti.
Ind.J.Exp.Biol., 15, 30, 1977.

59- Effect of *Crataeva nurvala* in experimental urolithiasis.
P. Varalashimi, Y. Shamila and E. Latha
J. Ethnopharmacol., 28, 313, 1990.

60- Studies of some herbal drugs used against kidney stones in Saudi folk medicine.
S.K. Aksar, A.H. Shah, M.O.M. Tanima, M.S. Ahmad, M. Tang and A.M. Aqeel.
Fitoterapia, 61, 435, 1990.

61- Effect of *Crataeva nurvala* on the biochemistry of the small intestine of normal and stone forming rats.
P. Varalashimi, E. Latha, Y. Shamila and S. Jayanthi.
J. Ethnopharmacol., 31, 67, 1991.

62- Distribution dynamics of cell surface associated cell CAM 105 in cultured rat hepatocytes.
A. Tingstrom and B. Obrink.
Exp. Cell Res., 185, 132, 1989.

Annexure XI

Establishment of an Animal House at Anadolu University

Medicinal Plants Research Centre, Turkey

1. Requirement of animals per month

Mice	100
Rats	100
G. Pigs	10
Rabbits	6

2.

Breeding stock required	Breeding females	Breeding males
Mice	50	25
Rats	100	20
Rabbits	10	2
G. Pigs	16	4

This will provide animals for research and also replacement stock for breeding.

3. Breeding method :

Mice: 2 females and 1 male to be kept in a cage permanently and only young one to be weaned at 21 days. The full cage to be discarded after one year or 6 to 7 litters which ever is earlier.

Rats: Four female and 1 male or 8 females and 2 males may be kept in a large cage. The pregnant females may be removed to a breeding cage at advanced pregnancy and allowed to litre there. The young ones may be weaned at 21 days and female returned to its original cage.

G. Pigs: Guinea pig can be kept in a pan in the ratio of 4 to 5 females and 1 male. At advanced pregnancy female may be removed to a breeding cage and returned to original pan after weaning of the young ones at four weeks.

Rabbit: Female is brought to the male cage for mating and removed immediately after the copulation. The female is kept in a seperate cage till weaning of the young ones at 6 weeks and can then be maked again.

4. Cage requirement:

The cages made of polypropylene/polycarbonate should be used for rat and mice and for guinea pigs if necessary. Stainless steel cages are recommended for rabbits. Size of cages and minimum number required for your breeding unit i.e. the breeding and growing stock is given below:

Size of the cage :

Mice	Approximately 30 cm x 25 cm x 14 cm is enough to keep two female and one male with litter till weaning. (No. required - 40).
Rat	Size 25 cm x 22 cm x 16 cm is enough to keep one breeding female with litter till weaning. (No required 40).
Rat flat cage	Size 60 cm x 45 cm x 16 cm is sufficient to maintain 10 to 12 adult females with 2-3 males for breeding (No required - 10).
Rabbit	Size 60 cm x 45 cm x 45 cm sufficient to keep one adult or 3 young & growing rabbits (No required - 10).
G.pigs	Pan size 75 cm x 75 cm x 25 cm is suitable to maintain 10 to 12 adult female guinea pigs with 2-3 males for breeding (No. required 5).
Breeding cage	45 cm x 30 cm x 30 cm is adequate to maintain a breeding G.Pig female with litter till weaning (No. required - 10).

Address of cage manufacturer :

M/s. Vishnu Traders
D-7, Industrial Estate
Roorkee - 247 667
(India)

Feed Commercial feed, if available, may be procured for the animals otherwise feed may be prepared using Soyabean meal, wheat, gram maize, fish, meal, milkpowder, supplemented with yeast, salt mineral mixture and vitamins to provide 20 to 22% protein as essential component.

Annexure XII

CARE OF LABORATORY ANIMALS

B. N. DHAWAN

Central Drug Research Institute

LUCKNOW 226001, INDIA

The laboratory animals provide the basic tool for all biological experiments and it is, therefore, necessary that they are of assured specifications and are also properly maintained, just as a chemist would like to be concerned about the quality and storage of chemicals. The animals, further, are living and hence need greater humane care and well known **ethical guidelines** (*Appendix I*) govern their care and use. In most countries guidelines for use and maintenance of laboratory animals are statutory requirements and are rigidly implemented, wherever this has not yet occured, it is bound to come soon. Even if these statutory requirements are not being enforced, the fundamental requirement of a biologist is to get reliable and reproducible results and hence the animal to animal variation needs to be minimised by using optimal husbandary, breeding and nutritional conditions conducive to good laboratory animal production and maintenance. This will also help in minimising the impact of anti-vivisectionist lobby considerably.

It is important that the animal facility is recognised as an independent entity, preferably housed in a **separate building** designed for this purpose and under the supervision of a qualified and trained veterinarian or biologist. The building, the environment and the cage are all capable of affecting the quality of animals and their performance. The animal house building needs to be provided with suitable barriers to control entry of contaminants and maintain separately clean and dirty areas. In a modern animal facility only 50-60% area is used for animal occupancy and the rest for various services including washing and sterilisation, food and cage store, quarantine, incinerator, monitoring laboratory, record room etc. The space requirements for commonly used laboratory animals have been summarized in Table 1. It may be mentioned that the food and water intake and therefore the growth and fertility. A 4°C change in temperature has been shown to cause 10-fold change in toxicity of some chemicals. A 12 hour light and darkness cycle with 300 Lux **illumination** at the floor level, **noise level** below 50 decibels and 10-15 **air changes/hour** in the animal rooms are also absolutely essential. In addition to the

"macro-environment", the **"micro-environment"** within the cage is also equally important. The cages should be of a suitable material (stainless steel, polypropylene etc.), size and shape to ensure not only a comfortable stay for the animal but also proper cleaning and adequate food and water supply.

The **breeding** of laboratory animals is a specialised job but has got to be undertaken in the laboratories in most developing countries due to non-availability of animals from commercial sources. A trained person is required for this job and it is essential that each species and strain is maintained in a separate room. The reproductive norms of commonly used laboratory animals are given in Table 2. Briefly, there are outbred and inbred strains. The **outbred strains**, like the Charles Foster or Sprague Dawley rat; the CF1 or Swiss mice,need a closed colony breeding but many animals should be random mated to maintain the outbred vigour. For most experiments the outbred animals are very suitable and easiest to be used and maintain. The **inbred strains** (Wistar rat, C57BL or Balb/C mice) require brother sister mating for at least 20 consecutive generations to achieve a very high cosanguinity factor. These strains have to be continuously monitored for selected markers, i.e., haemoglobin beta chain, esterases, skin graft etc. to ensure maintenance of inbred characteristics. They are useful for research in oncology, immunology, radiobiology etc. Some **special inbred strains** have been developed during the last few decades for very specific purposes. These include the Okamoto **Spantaneouly Hypertensive (SHR)** rat (where even the brother-sister mating has to be done between hypertensive litter mates only), its offshoot, the stroke prone SHR rat and the thymus deficient (athymic) **nude mouse**. Since these mice are T Cell deficient, they are particularly useful for production of monoclonals and for studying immunity during experimental infections. Many **hybrid strains** have also been developed for special purposes by crossing between the inbred strains, particularly of mouse. These are called F1 strains, are more resistant than inbred strains due to hybrid vigour, and are always produced by corss breeding only. Their main use is in oncology and immunology.

Another important aspect of maintaining a healthy animal colony is to ensure cleanliness and disease control by **microbiological monitoring** or at least to enforce adequate hygeinic measures including cage cleaning and sterilisation and food and water safety etc. This is adequate for most animal facilities producing and housing conventional **(Agnotobiotic)** animals. They do harbour many microorganism but good husbandary conditions ensure that no serious pathogenic organism is present. A variant of these animals are the **Specific Pathogen Free (SPF)** animals which are free of pathogenic organisms and require very special care and housing. The other specialized type of animals are known as **Gnotobiotic animals** and these may be germ free **(Axenic)** or microbially defined **(Gnotoxenic)**. Both require sterile conditions, are derived by hysterectomy and bred and maintained in isolators. They are required only for rigidly controlled immunological, transplant experiments etc. and need highly trained persons for management. They are also expensive to procure and maintain.

In any animal colony it is necessary to regularly monitor the health of the animals and the **haematological** and **serum biochemical parameters** are good indicators of health status, in absence of zoonotic infections, ectoparasites etc. A summary of normal values is given in Tables 3 and 4 respectively as a guide. This data should be generated in-house in the animal facility since there are many variables and a more important criterion is maintenance of values within the norms obtained in the concerned laboratory.

Attention was drawn in the very beginning to the ethical guidelines. Before concluding, it may again be stressed that laboratory animals must be treated humanely and with kindness. It must be assumed that procedures which would cause pain in human beings cause pain in other vertebrates, particularly mammals, as well. Procedures causing more than momentary pain or distress should be performed with appropriate sedation, analgesia or anaesthesia as per the norms of veterinary practice. Animals likely to suffer severe or chronic pain, discomfort or disability must be painlessly sacrificed at the end of the experiment.

A selected bibliography is provided in *Appendix II* to help obtain detailed information about proper breeding and management of Laboratory Animals. Some physiological norms have been given in Table 5.

Table 1: SPACE REQUIREMENTS FOR ADULT LABORATORY ANIMALS

Animal species	Weight	European Recommendations (*)		US Recommendations (**)	
		Floor area	Height	Floor area	Height
Mouse	20-25 g	180 cm2	12 cm	77 cm2	12.7 cm
Rat	300-400 g	350 cm2	14 cm	258 cm2	17.8 cm
Guinea Pig	> 350 g	600 cm2	18 cm	652 cm2	17.8 cm
Rabbit	2-4 kg	0.3 m3	35 m.	0.28 m2	35.6 cm

Adopted from:
(*) Official Journal of European Communities, December 1986
(**) NIH Guide for the Care and Use of Laboratory Animals No.85-23, 1985.

Table 2: REPRODUCTIVE DATA OF COMMONLY USED LABORATORY ANIMALS

Parameter	Mouse	Rat	Guinea Pig	Rabbit
Oestrus cycle (days)	4-5	4-5	16	-
Duration of Oestrus(hr)	10	13-15	6-11	Continuous
Time of Ovulation after Oestrus (hr)	2-3	3-10	10	Induced 10-11 hr after Coitus
Gestation Period (days)	21	21	68	30
Litter Size	10	3-12	1-4	4-5
Oestrus after Parturition	Post-partum	Post-partum	Post-partum	35 days
Reproductive Life span (years)	1	1	3-4	2-3
Mating System (Male:Female ratio)	1:2 Harem	Pair Harem	1:5 Harem	1:1 Hand Mating

Table 3: HEMATOLOGICAL DATA OF LABORATORY ANIMALS

Hematological Values	Mouse (*)	Rat (**)	Guinea Pig (***)	Rabbit (***)
WBC x 10(3)	8.5-10	7.5-14	8-9	5.2-7
RBC x 10(4)	6.4-6.6	6.5-9	4.6-5.6	4.6-5.4
Platelets 10(4)	108-27	39-74	104-638	18.9-53
Haematocrit	40	40-52	47-55	33-50
Neutrophil (%)	13 - 21	17 - 20	45 - 47	-
Eosinophil (%)	1 - 1	1 - 2	1 - 2	-
Basophil (%)	0	0	0	-
Monocyte (%)	0	0	1	-
Leucocyte (%)	79 - 86	78 - 81	51 - 52	-
Haemoglobin g/dl	17.3 - 17.4	8.88 - 11.2 mmol/L	4.3 - 15.1	-
Haemoglobin index	- - -	0.83 - 1.16	1.01	-
MCH picogram	17.4 - 17.9	0.97 - 1.42 mmol	28.4 - 29.0	-
MCHC %	32.7 - 34.1	18.9 - 26.8 mmol/L	34.2 - 34.5	-

(*) Adult NMRI Swiss
(**) Adult OFA (Sprague-Dawley)
(***) Adult albino

Table 4: BLOOD BIOCHEMICAL DATA OF MOUSE AND RAT

Biochemical Values	Mouse *		Rat **	
Sodium	153 - 154	mEq/L	137 - 154	mmol/L
Potassium	0.9 - 5.7	mEq/L	4.0 - 6.6	mmol/L
Chloride	113 - 115	mEq/L	99 - 108	mmol/L
Calcium	92 - 97	mg/L	2.3 - 2.8	mmol/L
Phosphates	92 - 95	mg/L	1.36 - 2.97	mmol/L
Glucose	0.19 - 1.14	g/L	4.5 - 8.95	mmol/L
Bilirubin	0.08 - 0.55	mg/L	0.51 - 6.67	mcmol/L
BUN (Urea)	-		4.32 - 12.95	mmol/L
Cholesterol	0.26 - 1.57	g/L	0.50 - 0.91	mmol/L
Total Bilirubin	1.3 - 4.8	mg/L	0.51 - 6.67	mcmol/L
Proteins	56 - 60	g/L	60 - 79	
Albumin	33 - 36	g/L	-	
Globulin	24	g/L	-	
Alb./Glob. Ratio	1.4 - 1.5		-	
Creatinine	3.6 - 4.2	mg/L	-	
Serum Alk. Phosphate	321 - 349	UI/L	71 - 299	mU/ml
SGOT(ASAT)	587- 618	UI/L	77 - 622	mU/ml
SGPT(ALAT)	138- 172	UI/L	28 - 418	mU/ml

(*) Adult NMRI Swiss
(**) Adult Sprague-Dawley

Table 5: PHYSIOLOGICAL NORMS OF COMMONLY USED LABORATORY ANIMALS

Parameter	Mouse	Rat	Guinea Pig	Rabbit
Weight at birth (g)	1 - 2	4 - 5	20	50
Age of weaning (weeks)	3	3	4	8*
Weight at weaning (g)	9 - 12	40 - 50	250	800
Maturity age (weeks)	8	12	20	24
Maturity wt. (g)	18 - 22	150 -200	250 - 400	1.5 -2.0 kg
Adult wt. (g)	25 - 30	200 - 300	400 - 500	2 - 2.5 kg
Respiratory rate/min.	160	200	80	55
Heart rate/min	600	300	150	130
Life span (years)	1.5 - 2	2.5 - 3	4 - 5	4 - 5
Rectal temperature (oC)	38.0	38.0	38.5	39.5

Colonies of all these animals can be best maintained at a room temperature of 20(+/-1)oC and humidity of 50%.

Appendix I.

ETHICAL PRINCIPLES IN ANIMAL EXPERIMENTATION

Basic Principles

Article 1

Progress in the field of human knowledge, and especially progress in biology, human and animal medicine is necessary.

Article 2

Man needs animals in his quest for knowledge just as he does to feed and clothe himself, and for his work. He thus has the duty to respect animals, his auxiliaries, who are living beings just as he himself is a living being.

Article 3

Every person involved in biological experimentation must recognize the fact that animals are gifted with sensitivity and memory, and that they have the capacity to suffer without any recourse to pain alleviating means.

The Responsibilities of the Investigators

Article 4

The investigator is on moral grounds responsible for his options and his acts within the field of animal experimentation.

Article 5

Experiments concerning living vertebrates and the collection of tissues on living subjects for research purposes must be performed by a *qualified biologist*, or under his direct supervision. The conditions within which the animals are maintained in the course of experimentation must be defined and monitored by a competent veterinarian or scientist.

Article 6

In the course of studies which involve the use of animals, a reasonable probability must prevail that the said studies will significantly contribute to the acquisition of knowledge which will in turn concur to improve the health and welfare of man and animals.

Article 7

Statistical methods, mathematical models and *in vitro* biological systems must be used when they are appropriate to complement animal experimantation, and to reduce the number of subjects used.

Article 8

The investigator must use the most suitable animal for his research, and he must also take into account the levels of sense and psychic awaresness which are inherent to each species. Animal species in danger of extinction, as defined within Annex I of the Convention on the International Trading of Animals in Danger of Extinction, will only be used under exceptional and well defined circumstances. As much as it is at all possible, animals used in laboratories will originate from specialized breeding outfits providing the best conditions of biological equilibrium.

Article 9

The investigator will make sure that the conditions under which the laboratory animals are kept are the best possible, and he must provide the required care to said animals before, during and after experiments.

Article 10

The investigator's duty is to spare the animals any physical or psychic pain which is not absolutely essential. He will implement methods which enable the limitation of suffering and pain whenever these conditions cannot be avoided.

[These ethical principles have been established in August 1979 by an International Group constituted at the outset of the International Symposium for "Laboratory Animals at the Service of Mankind" in Lyons, France.]

Appendix II

Selected Bibliography on Breeding and Care of Laboratory Animals

1. A.M.Allen and T.Nomura. Manual of Microbiologic Monitoring of Laboratory Animals. National Institutes of Health USA. Washington, 1986.

2. M.D. Buckland, L. Hall, A. Mowlem and B.F. Whatle. A Guide to Laboratory Animal Technology. Heinemann Medical Books, London, 1981.

3. M.E. Coates and B.S. Gustafeson. The Germ Free Animal in Biomedical Research. Laboratory Animals Ltd., London, 1984.

4. J.S. Fox, B.J. Cohen and F.M. Low (Eds.). Laboratory Animal Medicine. Academic Press, London, 1984.

5. T.B. Poole (Ed.) UFAW HANDBOOK ON THE CARE AND MANAGEMENT OF LABORATORY ANIMAL. 6th Edition, Longman Group, Marlow, 1987.

6. A. Speigel, S. Erichsen and H.A. Solleveld. Animal Quality and Models in Biomedical Research. Plenum Press, New York, 1985.

7. U.K. Stephens and N.M. Patton. Manual for Laboratory Animal Technicians. Publication no. 84-2, American Association for Laboratory Animal Science, Joliet, 1984.

8. U.S. Dept. of Health, Education and Welfare (DHEW) Publication No.80-23, Guide for the Care and Use of Laboratory Animals, NIH, Washington, Revised 1985.

Annexure XIII

LIST OF PARTICIPANTS

Prof. Bhola Nath Dhawan
Central Drug Research Institute, Lucknow, India

Prof.Dr. K.Hüsnü Can Başer
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Assoc.Prof.Dr. Yusuf Öztürk
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Süleyman Aydın
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Rana Arslan
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Nurhayat Ermin
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Yasemin Aktan
Anadolu University, Medicinal Plant Research Centre, Eskişehir, Turkey

Assoc.Prof.Dr. İpek Cingi
Anadolu University, Faculty of Medicine, Department of Pharmacology, Eskişehir Turkey

Assoc.Prof.Dr. Kevser Erol
Anadolu University, Faculty of Medicine, Department of Pharmacology, Eskişehir Turkey

Dr.Serdar Alpan
Anadolu University, Faculty of Medicine, Department of Pharmacology, Eskişehir Turkey

Assist.Prof.Dr. Mahmut Özdemir
Anadolu University, Faculty of Medicine, Department of Pharmacology, Eskişehir Turkey

Fatma Sultan Kılıç
Anadolu University, Faculty of Medicine, Department of Pharmacology, Eskişehir
Turkey

Yasemin Aydın
Anadolu University, Faculty of Medicine, Department of Physiology, Eskişehir
Turkey

Assoc.Prof.Dr. Erdem Yeşilada
Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey

Dr. Serap Gür
University of Ankara, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey

Assist.Prof.Dr. Mert Ülgen
Marmara University, Faculty of Pharmacy, İstanbul, Turkey

Dr. Sena Sezen
Marmara University, Faculty of Pharmacy, İstanbul, Turkey

Annexure XIV

WORKSHOP ON PHARMACOLOGICAL TECHNIQUES FOR STUDY OF NATURAL PRODUCTS
January 12-15, 1993

PROGRAMME

January 12, 1993		
9:00 AM		Inaugural Session Welcome - Prof. K.H.C. Başer Genesis and purpose of workshop - Prof. K.H.C. Başer
9:30 AM	Lecture 1.	Collection and processing of plant materials for biological testing Prof. K.H.C. Başer
10.00 AM	Lecture 2.	Organisation of a biological screening programme Prof. B.N. Dhawan*
1:30 PM	Lecture 3.	Screening of plant extracts for CNS effects and acute toxicity Prof. B.N. Dhawan
3:00 PM		Demonstration 1: Gross effects of drugs in mice Prof. B.N. Dhawan, Mr. S. Aydın, Miss R. Arslan
4:30		Discussion
January 13, 1993		
9:00 AM	Lecture 4.	Activities of TBAM - A Review Prof. K.H.C. Başer
10:00 AM	Lecture 5.	Use of smooth muscles of gastrointestinal tract for screening of plant extracts Dr. Y. Öztürk
11:00 AM	Lecture 6.	Screening plant extracts for cardiovascular effects Prof. B.N. Dhawan
2:30 PM		Demonstration 2: Effect of drugs on intestinal smooth muscle G. Pig ileum Rat duodenum Dr. Y. Öztürk, Mr. S. Aydın, Miss R. Arslan
4:30 PM	Lecture 7.	Spasmolytic activity of some natural products Prof. B.N. Dhawan
5:00 PM		Discussion

*UNIDO Consultant

January 14, 1993		
9:00 AM	Lecture 8.	CDRI research on medicinal plants Prof. B.N. Dhawan
10:30 AM	Lecture 9.	Use of other *in vitro* muscle preparations, for study of plant extracts Dr. Y. Öztürk
11:00 AM		Visit to TBAM laboratories
2:30 PM		Demonstration 3: Drug effects on CNS - Analgesia - Anticonvulsant activity Prof. B.N. Dhawan, Mr. S. Aydın, Miss R. Arslan
4:30 PM		Discussion
January 15, 1993		
9:00 AM	Lecture 10.	Hepatoprotective activity of natural products- Experimental evaluation Prof. B.N. Dhawan
10:00 AM		Brief presentations on their research activities by participants
2.30 PM	Lecture 11.	Thrust areas for medicinal plants research Prof. B.N. Dhawan
3:00 PM		Demonstration 4: *in vitro* preparations G. Pig auricle Rat uterus Dr. Y. Öztürk, Mr. S. Aydın, Miss R. Arslan
4:30 PM		Concluding Session Address by Prof. K.H.C. Başer Distribution of Certificates

Backstopping Officer's Technical Comments
based on the work of Mr. B.N. Dhawan
DP/TUR/88/001/11-03

The report describes in detail the range of activities carried out by the consultant. The consultant has organized and assisted the setting up of the pharmacological testing laboratory and advised on the plans for a new animal house. The need for training of counterpart staff in this field has been stressed.

In addition to commissioning of equipment, counterpart staff have been trained in the techniques. Protocols have been developed for specific activity evaluations. The Centre is now in a position to carry out the initial tests for screening the indigenous medicinal plants for a wide range of activities. The consultant has compiled a manual on activity testing techniques and demonstrated the use of some tests at a workshop for counterpart staff and scientists of other institutions. This manual will be further elaborated by the consultant as a field manual for use in other projects.

The consultant has also presented a large number of publications and books to the Centre. The Backstopping Officer fully agrees with the recommendations made by the consultant with regard to programme of future work, training and equipment needs and the development of an animal house and a herbarium.

The implementation of the recommendations on a priority basis will lead to further development of the Centre and achievement of the objectives of the project. The financial needs for these activities should be favourably considered by the government and the UNDP.