Higher Education Commission of Pakistan

Project Proposal Case 01

HIGHER EDUCATION COMMISSION

H-9, Islamabad (Pakistan)

For HEC use only
Proposal Identification Number

RESEARCH GRANT APPLICATION FORM

COVER SHEET FOR PROPOSAL

A. TITLE OF PROPOSED PROJECT **Studies on Antiglycation Agents from Natural Sources- A New Approach towards Molecular Treatment of Diabetes**

B. WHETHER PROPOSED RESEARCH IS BASIC ✓ OR APPLIED ☐

C1. RESEARCH DOMAIN

✓ Sciences ☐ Engineering & Technology ☐ Social Sciences ☐ Humanities

C2. STATE FIELD OF RESEARCH AND SPECIALIZATION (For example; Major: Chemistry, Specialization: Organic)

Major **Chemistry** Specialization **Bioorganic Chemistry**

D. PROJECT DIGEST. Describe the proposed research using (about 250) words geared to the non-specialist reader.

Diabetes mellitus and its complications are a huge health problem in Pakistan, particularly in disadvantaged communities, because of their poor access to modern healthcare facilities. Many individuals therefore rely on non-traditional medications such as plant extracts. It has been estimated that of 250,000 plants, less than 2% have been screened pharmacologically and even fewer for their anti-diabetic activity. This project will set up a program to screen and identify existing and novel natural products for their anti-glycation properties. Such products will undergo further evaluation for their therapeutic potential and may protect against diabetic complications. An attempt will be made to secure patents for promising compounds and their development into drugs in collaboration with national and international pharmaceutical companies by using the HEJ (Pakistan) and UK links.

This project is collaborative in nature and will allow an exchange of knowledge and expertise in a new area of research between a leading Pakistani institution and an established UK laboratory. The UK group will have access to structurally novel and pharmaceutically potent plant metabolites via the Pakistani group. Conversely, the Pakistani group will have access to expertise, training, literature and equipment at the Manchester Metropolitan University in the field of anti-diabetic protein glycation chemistry. Pakistan has a history of using plant material for medicinal purposes and the HEJ Institute has isolated over 3000 natural products. However, there is a need to assess scientifically whether these compounds can be used to develop marketable drugs with therapeutic potential. The current project investigates a novel area with little existing knowledge.

E1. PRINCIPAL INVESTIGATOR NAME (full with no initials)	E2. HIGHEST DEGREE	E3. POSITION/TITLE

E4. DEPARTMENT/SECTION	E5.UNIVERSITY/INSTITUTION	E6. MAILING ADDRESS
H.E.J. Research Institute of Chemistry, International Center For Chemical Sciences.	University of Karachi / H.E.J. Research Institute	H.E.J. Research Institute of Chemistry, International Center For Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

PROJECT DETAILS

1. PROJECT SUMMARY

Describe the proposed research using (about 250) words.

Diabetes is potentially one of the largest threats in the new millennium and severely affected the developing world. Diabetes is a disease appears due to the failure of the pancreases to produce insulin in enough quantity or any body system irregularity to utilize it effectively. The high blood glucose level is the key symptom of uncontrolled disease and results in serious damages and harms to different body systems including nerves and characterized by several complications like cataract, retinopathy, neuropathy, atherosclerosis, nephropathy and impaired wound healing.

Most of these complications are related to standard changes in tissues proteins by their non-enzymatic bindings with sugars molecules. About 5% of global of total global deaths are due to diabetes. Current treatments of diabetes are largely ineffective in achieving normal sugar levels and delaying the onset of late diabetic complication. Therefore, there is an urgent need for new strategies to cure from diabetes and the complications associated with it.

In current times, toxic drugs for chronic illnesses are increasingly unwelcome and people are looking for viable alternatives, herbal plants and natural products are considered as treasures of medicines. Many plants and herbs known to have antiglycation properties. Plants contain hundreds of chemicals. However few of them are responsible of pharmacological activities. For this purpose, a bioassay-guided isolation of active constituents of medicinal plants will be carried out by employing reliable bioassay models.

2. PROPOSED GOALS/OBJECTIVES (please identify quantifiable goals)

i. If the proposed research is basic, please identify or postulate scientific hypothesis on which your proposed goal is based.
ii. If the proposed research is applied, please clearly identify the output in the form of a product or process, need or relationship to industry and also identify the end-user of your output/ product. P.I. is encouraged to make preliminary inquiries with the proposed end user and attach any certificate/ document in support of the proposed research.

HYPOTHESIS/BASIS OF RESEARCH (if basic research)

Hyperglycemia, a complication associated with diabetes is considered as a key for the molecular basis of diabetic. During the process of glycation the sugar react with protein or nuclic acid to form glycation products, commonly known as Amadori or fructoseamine which further oxidized to Alpha-dicarbonyl compounds such as methylglyoxal, deoxyglucosone, and glyoxal (AGE precursors). These oxidized products are found to be more reactive and showed sevarl inter- and intramolecular interactions to form stable end products known as advanced Maillard products or advanced glycation end products (AGEs).

On the basis of the understanding of the whole glycation procedure, it is concluded that it is possible to stop the process of formation of AGEs at any stage of the reaction due to the availability of a wide range of electrophilic, neucleophilic and radical scavengers.

The compounds named aminoguanidine and aspirin are reported to have significant antiglycation potential, but showed some toxic effects.

Alteons are a new class of pharmaceutical compounds that can restore the cardiovascular system by disconnecting the AGE crosslink. The Alteon ALT-711 (3-phenacyl-4, 5-dimethylthiazolium chloride) is known as AGE breaker which inserts itself into AGE crosslinks and release the proteins. They are in the trail process. So there is need to discover some more AGEs breakers with potent activity and less toxicity.

Glycation of proteins in hyperglycemia becomes a therapeutic target for the treatment of diabetes. The growing

interest in the field of natural products is due to their low toxicity. This growing interest of health researchers in natural products and their derivatives in the main stimulus for us to focus our efforts in this filed to identify natural antiglycation compounds and to carryout structural modifications for their improved antiglycation abilities. The major goal of this project is aimed at the discovery of active constituents from medicinal plant, which are use traditionally by indigenous people for curing the diabetes.

GOALS/OBJECTIVES (please quantify your objectives in case of Applied research)

The aim of this project is to identify natural chemicals derived from plant sources in Pakistan that can be utilised for the treatment of diabetic complications. The objectives are:

- To screen selected natural products for their ability to inhibit formation of sugar-modified proteins called advanced glycation endproducts.
- To investigate whether these natural products or their constituents can protect cells against the side effects of high blood sugar and advanced glycation endproducts.
- To unravel the mechanism and site of action of promising compounds.

This work will further strengthen existing links between a UK and Pakistani laboratory engaged in high quality research.

IDENTIFY END USER/ BENEFICIARY INDUSTRY (if applied research)

The results of the study will be extensively evaluated in In-Vivo animal models. The successful antiglycation agents will be further developed through various phases of clinical studies in collaboration with local pharmaceutical industry.

3. **INTRODUCTION** (not to exceed one page)

The introduction should consist of three paragraphs; the first paragraph should indicate the scientific hypothesis/commercial basis on which the project is based. The second paragraph should introduce the precise nature of the project, and the final paragraph should indicate the proposed objectives in the light of the first two paragraphs and explain clearly what the reader will see in the main body of the proposal.

Diabetes mellitus is a common disease where affected patients have high blood sugar levels. It is a serious health problem world-wide because affected people suffer from damage to their eyes, skin, kidneys, nerves and blood vessels. It is generally accepted that the damage observed in diabetic patients is because of the high blood sugar levels. Blood sugar can react with body proteins during a process known as glycation to form structures called Amadori products. These Amadori products can react with other proteins and sugars, forming complex cross-linked molecules known as advanced glycation endproducts (AGEs). These AGEs build-up in body tissues altering their function and can also react with and damage body cells, particularly cells lining blood vessels called endothelial cells. It is believed that several damages observed in the diabetic patients are due to these AGEs.
Pakistan has a history of using plant products for medicinal purposes, as they often have less side effects and the H. E. J. Research Institute of Chemistry in Karachi has isolated over 3000 new substances. The aim of this project will be to find new chemicals that can prevent formation of AGEs and can protect the cells from the effects associated with the end products of glycation. Proteins will be incubated with sugars for a defined period of time so that glycation takes place and the resultant AGEs measured by their effect on protein cross-linking. Selected natural products will be included in this incubation mixture to see how effective they are in inhibiting formation of AGEs. High sugar levels and AGEs are known to damage endothelial cells causing a reduction in their number. Endothelial

cells will be grown artificially and exposed to high sugar and AGEs with and without selected natural products. The cells will be counted after a defined period of time to see whether the natural product offers any protection against the high blood glucose level. Any natural product that has protective effects for both proteins and cells will be studied in detail to determine how it acts and whether before or after Amadori product formation.
On successful completion, this project should identify a number of new plant-derived substances that can protect us against the harmful effects of high blood sugar. Such substances might be of use in the treatment of diabetes mellitus and protect against the damage caused by high blood sugar.
This collaborative project will utilize and combine the expertise of a UK laboratory engaged in diabetes research with a Pakistani laboratory engaged in the isolation and characterization of plant extracts.

4A. BACKGROUND OF THE RESEARCH PROBLEMS TO BE ADDRESSED (Not to exceed two pages)

i. In case of basic research, a comprehensive and up-to-date literature survey clearly highlighting the existing gaps and what new information will be added to the existing pool of knowledge.
ii. In case of applied research, please also identify the industry in Pakistan, which should benefit from the process/product. Please justify how the proposed research will contribute to the national economy/social sector. Please justify your claim by giving figures of import/export, present market, future trends etc. The principal investigator is encouraged to discuss the proposed research with the proposed beneficiary and attach supporting documentation.

Common disorders like hyperglycemia and predisposes are associated with diabetes mellitus and afflicting the eyes, nerves, blood vessels and kidneys. During hyperglycaemia, glucose and other reducing sugars can react with amino functionality of the protein to form Schiff base which intum rearranges to more stable Amadori product. This glycated protein undergoes further reactions to form cross-linked and fluorescent structures, called advanced glycation endproducts (AGEs).
Circulating serum AGEs can damage endothelial cells *via* interaction with receptors for AGEs (termed RAGE) and this is believed to underlie the pathogenesis of diabetic vascular complications. There is interest in compounds that can prevent AGE formation because of their therapeutic potential. Much attention has focused on synthetic compounds but there is growing interest in natural products. Recently, several products, isolated from plants, have been shown to possess anti-glycation properties. Such products may be use to prevent from the diabetes complications before their onset.

Aims and Objectives
The overall aim of this project is to screen selected natural products in order to identify those with anti-glycation properties. The specific objectives are:

- To screen selected natural products in order to determine whether they inhibit the formation of AGEs.
- To investigate whether identified natural products with anti-glycation properties can protect cultured endothelial cells against hyperglycaemia and AGE-mediated toxicity.
- To investigate the mechanism of action of natural products with anti-glycation properties.

Experimental

The experimental work will be divided into three stages:

(1) Natural products with a history of anti-diabetic use will be selected and screened for their ability to reduce AGE formation *in vitro*. Crude extracts will be screened initially, although the ultimate goal is to identify active ingredients. Model proteins will be glycated *in vitro* in the presence or absence of the selected natural products and the formation of AGEs will detected by their protein cross-linking on sodium dodecyl polyacrylamide gel electrophoresis.

(2)The ability of selected natural products to protect cultured endothelial cells against the harmful effects of high

glucose and added AGEs will be investigated. Bovine serum albumin (BSA) will be glycated by incubating with glucose to produce AGEs. Endothelial cells will be grown in culture in the presence of high glucose and added BSA-AGEs which inhibit the cellular proliferation. Selected natural products will be included in the culture medium to see whether they protect against the anti-proliferative effects of high glucose and AGEs. The extent of proliferation will be determined by direct counting using a Coulter counter. The methodology required has already been established in the UK laboratory.

(3) Those natural products capable of inhibiting AGEs will be investigated further for their mechanisms of action ie whether the inhibitory effect is pre- or post-Amadori. During glycation, free protein amino groups decrease whereas Amadori groups increase. Measurement of these groups when proteins are glycated in the presence of natural products may indicate whether the inhibitory effect is pre-Amadori. Protein glycation is accompanied by generation of protein-bound carbonyl groups and previous studies have shown that certain anti-glycation compounds react with glycated proteins causing a reduction in protein-bound carbonyl groups and therefore AGE formation. Anti-glycation compounds containing amino groups may react by blocking carbonyl groups on Amadori products and AGEs thus changing ketone to imino groups. The effect of selected natural products on post-Amadori reactions involved in AGE formation will also be investigated. This will be achieved by reincubation of dialysed glycated proteins in the presence of natural products and subsequent quantification of AGEs. Methods for measurement of free amino groups, Amadori adducts, imino groups, protein-bound carbonyl groups and monitoring of post-Amadori AGE formation are established and in use in Dr. Ahmed's laboratory at the Manchester Metropolitan University.

In summary, this research project will allow us to screen selected natural products for their anti-glycation properties and help to identify those with therapeutic potential.

4B. RESEARCH PLAN: SCHEDULE/PHASING (Not to exceed one page)

Year 1 and 2: Identification, Selection and screening of natural products for their anti-glycation activity will be conducted at the HEJ Research Institute of Chemistry in Karachi. Model proteins (eg lysozyme) will be glycated by incubation in glucose with and without selected natural products. The AGEs will be monitored by their cross-linking on gel electrophoresis following Coomassie blue staining. This screening will identify compounds capable of inhibiting the formation of AGEs *in vitro*.

Year 2 and 3: Selected natural products with anti-glycation activity will be evaluated further to see whether they can protect cultured endothelial cells against anti-proliferative effects of high glucose and AGEs. Promising compounds will be studied further to gain an insight into their mechanism and site of action and their toxicity or IC_{50} values will be determined. This work will be conducted at the Manchester Metropolitan University, UK, and aim to identify suitable anti-glycation compounds.

On successful completion of this project, those compounds with anti-glycation properties will be investigated further for their toxicity. Promising compounds may undergo *in vivo* studies in diabetic animals and then ultimately humans to determine their therapeutic potential, possibly involving collaboration with relevant industrial partners

5. **IMPACT** (of proposed research on teaching/training of manpower, institutional capability building and on local industry)

- The proposed project is to minimize complications and maximize quality of life of diabatic patients.
- Workshops on diabetes will be organized, which provide forums for reviewing most recent scientific development and providing guidance for implementation to improve prevention and health care.
- To produce disseminate a new scientifically-based review on the prevention and complication of diabetes.
- To produce up-to-date, practical guidance for policy makers of Pakistan, on the contents, structure and implementation of national diabetes program.
- Researchers who will be trained in United Kingdom during this project will establish the similar experimental set up in Pakistan and educate their colleagues so the process will continue in terms of capacity building.

6. **COLLABORATING LABS**

In case of collaboration with national/international research group or local industry, please identify clearly the parts of research that will be carried out in the participating laboratories and please identify complimentarity and/or justify the need for collaboration) P.I.s are encouraged to find collaborating partners within Pakistan, particularly in less developed areas. Include a letter from Collaborating agency expressing willingness to collaborate.

H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

Department of Biological Sciences, The Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK.

Responsibilities Pakistan Partner	Responsibilities UK Partner
• Collection of Data/ Purchase of Chemicals	• *In vivo* Studies
• Collection/Purchase of Plants/Extract Preparation • Screening of antiglycation properties	• Toxicological Profile
• Phytochemicals Studies • Structural Elucidation	• Study of Mechanism
• *In vitro* Studies Bioassay of Plant Extracts/Pure Compounds	• Publications/ Patenting
• Chemical Derivatization • Toxicological Profile of Potent Inhibitors	
• *In vivo* on Animal Model and Evaluation of Some Potent Inhibitors	
• Publications/ Patenting	

7. FACILITIES AND FUNDING

7A. Facilities: equipment available for the research project IN THE HOST UNIVERSITY/INSTITUTION

7B. Scientific Personnel

a. Available

b. Required*

1. Research Officers (2)
2. Lab Assistant (1)

*Involvement of research students is encouraged.

7C. Other funding available for the proposed studies (if any)

No funding available

8. PRINCIPAL INVESTIGATOR

A brief resume of research accomplished in the last 05 years. Please specify title of the research proposal(s), duration, funding source(s) and award amount(s).

CV Attached

8. PRINCIPAL INVESTIGATOR: continued

1.	Please attach C.V.	
2.	Number of Publications during the last five years & page numbers on the C.V. where these publications are listed	National: __ _____Please see pages_____ of CV International: __555 Please see pages : _____ of CV
3.	Number of research projects completed & page number where this information appears	Basic: _____ Please see pages _____ of CV Applied: _____ Please see pages_____ of CV

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD

DESCRIPTION	% of time devoted to Project	YEAR 1 (Rs. In million)	YEAR 2 (Rs. In million)	YEAR 3	Amount (in million Rs.)
A. Salaries and Honorarium					
PI: One month/year of basic pay @ 12000 / month		0.144	0.144		0.288
Co-PI: One month basic pay for the entire duration @ 12000/ month		0.144	0.144		0.288
Research Officers (2) (each @ 8000/ month)		0.192	0.192		0.384
Lab Assistant (1) @ 4000/ month		0.048	0.048		0.096
Subtotal:		0.528	0.528		1.056

B. Permanent Equipment (Please attach invoice/quotation and expected delivery date for items costing over Rs. 0.1 million.)				
Not Required				
Subtotal:				

C. Expendable supplies *(year wise quantity with full justification)*				
Consumable				
Plant material	0.1	0.1	0.1	0.3
Solvents for chromatography and spectroscopy	0.2	0.2	0.1	0.5
Bioassay supplies including animals	0.2	0.2	0.3	0.7
Disposable items	0.2	0.2	0.1	0.5
Subtotal:	0.7	0.7	0.6	4.0

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD (continued)

DESCRIPTION	YEAR 1	YEAR 2	YEAR 3	Amount (in million Rs.)
D. Others D1. Literature, documentation, information, online literature search, contingencies, postage, etc.				
Literature/ Reprints	0.025	0.015	0.01	0.050
Postage	0.003	0.003	0.004	0.01
Contingencies	0.04	0.03	0.03	0.10
Subtotal:	0.068	0.048	0.044	0.16

D2. Local Travel (Destination and purpose with full justification)				
Presentation in National Conference	0.025	0.025		0.05
Subtotal:				

D3. Miscellaneous				
Audit Fee (Max. Rs 10,000)				0.01
Accountant Fee (Max. Rs. 10,000)				0.01
Subtotal:				0.02
Subtotal (D1 + D2 + D3):				0.18
E. Indirect cost (University overheads) 02 % of Total direct cost to meet office support, utilities, etc.				
Grand Total (A + B + C + D+E):	2.633	2.633		5.286

9B. JUSTIFICATION (Please justify your request in a background of the existing facilities available at the host Institute.)

	Description	Justification
A	**Salaries & Allowances**	
1	Research Officers (1): For bioassay-guided fractionation, isolation and structure elucidation of secondary metabolites and for the bioassays Research Officers (1): For bioassays	Qualification: Masters in organic Chemistry (Ph.D. student) at least three first division in the education carrier Qualification: Masters Biochemistry, at least three first division in the education carrier.
2	Lab Attendant to maintain the lab	Qualification: Matriculation Experience:2-3 years experience in laboratory.
B	**Permanent Equipment**	Not applicable
C	**Expendable supplies**	
1	Plant Material (5-8 Kg of dry weight of each)	For phytochemical investigations
2	Solvents (50-80 L each) EtOH MeOH Hexane/Pet. Ether CH_2Cl_2 EtOAc BuOH	For extraction and purification of natural products
	HPLC grade solvents (30-50 L each) MeOH H_2O Acetonitrile	For purification of natural products
	Deutrated solvents (5-10 L each)	For NMR experiments (Structure elucidation)
3	Chemicals and media Bovine serum albumin (BSA) glucose anhydrous trichloroacetic acid (TCA) Sodium azide (NaN_3) Dimethyl sulfoxide (DMSO)	Chemicals to check the anti-glycation effects of medicinal plant extracts and pure compounds both *in-vitro* and *in-vivo*.

	Sodium dihydrogen phosphate (NaH_2PO_4) Sodium chloride (NaCl) Disodium hydrogen phosphate (Na_2HPO_4) Potassium chloride (KCl) Potassium dihydrogen phosphate (KH_2PO_4) Sodium hydroxide (NaOH) Will be purchased from Sigma Aldrich .	
4	Animal studies (including BALB, Mice, Cages, feeding tubes, restners, etc)	*In-vivo* anti diabatic studies will be conducted on mice and rats models.
5	Dispossible items (Vials, tubes, botteles, Pipettes, etc)	For Isolation and purification of bioactive natural products and for bioassays
D	**Others**	
D1	Literature, documentation, information, online literature search, contingencies, postage, etc.	To study the recent international development related to the subject and to process the publications and papers.
D2	Travel	PI and CO-PI will be travel to participate in conferences and symposium related to Project.
D3	Miscellaneous	
	Accountant Audit fee	To maintain the account Audit will be conducted to make sure the proper utilization of the fund.

Project Proposal Case 02

HIGHER EDUCATION COMMISSION

H-9, Islamabad (Pakistan)

For HEC use only
Proposal Identification Number

RESEARCH GRANT APPLICATION FORM
COVER SHEET FOR PROPOSAL

A. TITLE OF PROPOSED PROJECT

High Resolution X-Ray Analysis of Pharmaceuticals by Important Enzymes in Complex with Plant-Based Inhibitors as a Basis for Rational Drug Design

B. WHETHER PROPOSED RESEARCH IS BASIC ✔ OR APPLIED ☐

C1. RESEARCH DOMAIN

✔ Sciences ☐ Engineering & Technology. ☐ Social Sciences ☐ Humanities

C2. STATE FIELD OF RESEARCH AND SPECIALIZATION (For example; Major: Chemistry, Specialization: Organic)

Major Chemistry Specialization Bioorganic Chemistry

D. PROJECT DIGEST. Describe the proposed research using (about 250) words geared to the non-specialist reader.

The understanding of diseases at a molecular level enables scientists to develop structure-based drugs. In terms of the project proposed here, natural (Plant-based) inhibitors of medicinally important enzymes is identified by applying mechanism-based *in-vitro* screening methods. Their mechanism of action will be studied by employing enzyme kinetics protocols. More importantly, selected enzyme-inhibitor complexes will be crystallized and their structures will be studied by single-crystal diffraction techniques using synchrotron radiations up to high resolution.

This project will be the first of its type in Pakistan and will lead to the identification of effective inhibitors of medical relevant and important enzymes. This structure-based approach towards the drug discovery will not only yield a better understanding of the inhibition mechanism, but will also support the identification of bioactive molecules to be further developed as effective leads and drugs to treat enzyme-related diseases.

E1. PRINCIPAL INVESTIGATOR NAME (full with no initials)		E2. HIGHEST DEGREE	E3. POSITION/TITLE
E4. DEPARTMENT/SECTION **H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences**	E5. UNIVERSITY/INSTITUTION **University of Karachi**	E6. MAILING ADDRESS **H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan**	

PROJECT DETAILS

1. PROJECT SUMMARY

Describe the proposed research using (about 250) words.

Non-covalent interactions between the proteins and other molecules play an important role in biological processes. X-Ray crystallography and NMR spectroscopy are the two main techniques employed to characterize the mechanism of these interactions and the structures of the binding sites. The main aim of modern drug development is to design molecules which can interact with the active sites of an enzymes to prevent the bio-chemical reaction and as a result, stop the progress of a particular disease.

To realize the promise of modern drug discovery, new methods are needed to characterize both the three dimensional structures and active site of proteins.

The proposed study entails three-dimensional crystallographic structural determination of medicinally important enzymes, and their complexes with known inhibitors. Structural detail of the enzyme-inhibitor complex will reveal the conformational changes in the enzyme that aid to understand the inhibition mechanism. Likewise, identification of important enzyme-inhibitor interactions will enable *de novo* design of new inhibitors that optimize those interactions. This will lead to an inhibitor with high potency and specificity which leads to a drug with low toxicity and least side effects.

2. PROPOSED GOALS/OBJECTIVES (please identify quantifiable goals)

i. If the proposed research is basic, please identify or postulate scientific hypothesis on which your proposed goal is based.
ii. If the proposed research is applied, please clearly identify the output in the form of a product or process, need or relationship to industry and also identify the end-user of your output/ product. P.I. is encouraged to make preliminary inquiries with the proposed end user and attach any certificate/ document in support of the proposed research.

HYPOTHESIS/BASIS OF RESEARCH (if basic research)

Plant-based compounds are structurally diverse and in-principal capable of locking a particular disease mechanism. Their capacity to reversibly modulate the function of a clinically important enzyme can be exploited to develop effective medicines. During this project, this hypothesis will be checked for selected clinically relevant enzymes.

GOALS/OBJECTIVES (please quantify your objectives in case of Applied research)

The specific objectives of the proposed study are as follows:

1. To study the non-covalent interactions of libraries of natural products and their synthetic derivatives on the formation of complexes with different biologically important enzyme by using X-ray crystallography.
2. To unravel the mechanism and site of action of promising compounds, and use this information to initiate rational design of new (more potent/selected) drugs.
3. To develop productive research collaboration between the two institutions in the field of rational drug designing.

IDENTIFY END USER/ BENEFICIARY INDUSTRY (if applied research)

3. INTRODUCTION (not to exceed one page)

The introduction should consist of three paragraphs; the first paragraph should indicate the scientific hypothesis/commercial basis on which the project is based. The second paragraph should introduce the precise nature of the project, and the final paragraph should indicate the proposed objectives in the light of the first two paragraphs and explain clearly what the reader will see in the main body of the proposal.

Enzyme inhibitors play a significant role in the preservation of human health. Our preliminary search for plant-based inhibitors has already led to the discovery of a number of naturally occurring compounds that may be used in the treatment of a variety of ailments. These components possess inhibitory effects against three pharmaceutically important enzymes: Glycosidase, Cholinesterase, and Urease. A number of studies have identified a central role of these enzymes in diseases, like Alzheimer's disease, hyperglycemia, or infectious diseases caused by pathogenic bacteria. Specific inhibition of these key enzymes is an important area of pharmaceutical research.

Crystallography is a branch of structural biology which is considered as the central technique to design the structure-based drug molecules. Our German collaborators have access to high intensity synchrotron radiation provided by DESY Hamburg, at the University of Hamburg that enable them to determine the 3-D-structures of the relevant enzymes and their complexes with the inhibitors. In collaboration with them, we will examine the structures and identify critical inhibitor-protein interactions, and use this information to synthesize and design novel inhibitors that optimize those non-covalent binding interactions for a greater efficacy.

(PLEASE ATTACH ONE SHEET ONLY)

4A. BACKGROUND OF THE RESEARCH PROBLEMS TO BE ADDRESSED (Not to exceed two pages)

i. In case of basic research, a comprehensive and up-to-date literature survey clearly highlighting the existing gaps and what new information will be added to the existing pool of knowledge.
ii. In case of applied research, please also identify the industry in Pakistan, which should benefit from the process/product. Please justify how the proposed research will contribute to the national economy/social sector. Please justify your claim by giving figures of import/export, present market, future trends etc. The principal Investigator is encouraged to discuss the proposed research with the proposed beneficiary and attach supporting documentation.

The Higher Education Commission of Pakistan (HEC) and Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) have jointly sponsored the visit of Pakistani scientists to various science institutions of Germany during May 23-May 30, 2008. The delegation was asked to come up with practical proposals to initiate joint research projects between the two countries. With this spirit, the Pakistani scientists discussed various research projects with their German counterparts. One of this project was to initiate structure-based drug designing in Pakistan by using X-ray diffraction techniques. This project is now presented for the approval of the HEC, Pakistan.

The project is based on the following three very important enzymes α-glucosidase, Urease and choleinesterase.

1. The last and final step during the digestion of carbohydrates is catalyzed by α-glucosidase enzyme. The inhibitors of α-glucosidase significantly reduces the uptake of carbohydrates with suppression of hyperglycemia and therefore can be utilized to treat the obesity and diabetes patients (Ashry et al., 2000). The α-glucosidase inhibitors are known to lower the insulin requirements and are suggested as antiobesity drugs, immune modulator, and insect antifedant and fungicidal in nature. Two novel and significant α-glucosidase inhibitors, named epiexcelsin (IC_{50} = 59.8 μM) and 5'-demethoxyepiexcelsin (IC_{50} = 75.2 μM) have been isolated (Abbasi et al., 2005) from *Commiphora mukul* (Guggulu), a medicinally important plant with a wide range of uses in indigenous systems.
2. Urease: Ureolytic bacteria are associated with several pathologies including urinary tract infections, hepatic encephalopathy, hepatic coma, and polynephritis. *Helicobacter pylori* is the major causative agent of gastrodudonal infection (Mobley et al., 1989; 1995). Therefore, there is a need to improve the therapeutic potential against the infection due to ureolytic bacteria as the hydrolysis of urea by these organisms is responsible to provide them the suitable environment for survival (Mobley et al., 1989; 1995). Biscoumarins

are the natural products with a wide range of biological activities (Sengupta et al., 1985 Murray, 1991), have been identified to be efficient inhibitors of urease (Khan et al., 2003).

3. Cholinesterase: Acetylcholinesterase (AChE), the key enzyme to treat the Alzheimer disease catalyzes the acetylcholine hydrolysis (Quinn, 1987) and therefore identified as target to design the inhibitors. A number of studies shown that during early stage of Alzheimer's diseases the aggregation of β-amyloid peptide is accelerated by AChE and the non-compitative inhibitors of AChE can inhibit this aggregation process. However non-competitive inhibitors are proved to be failed (Yu et al., 1999, Nicolet et al., 2003). Three triterpenoidal alkaloids from medicinally important plant, *Buxus papillosa* (from North West Frontier Province of Pakistan) are found to be anticholinesterase in nature (Atta-ur-Rahman et al., 2001).

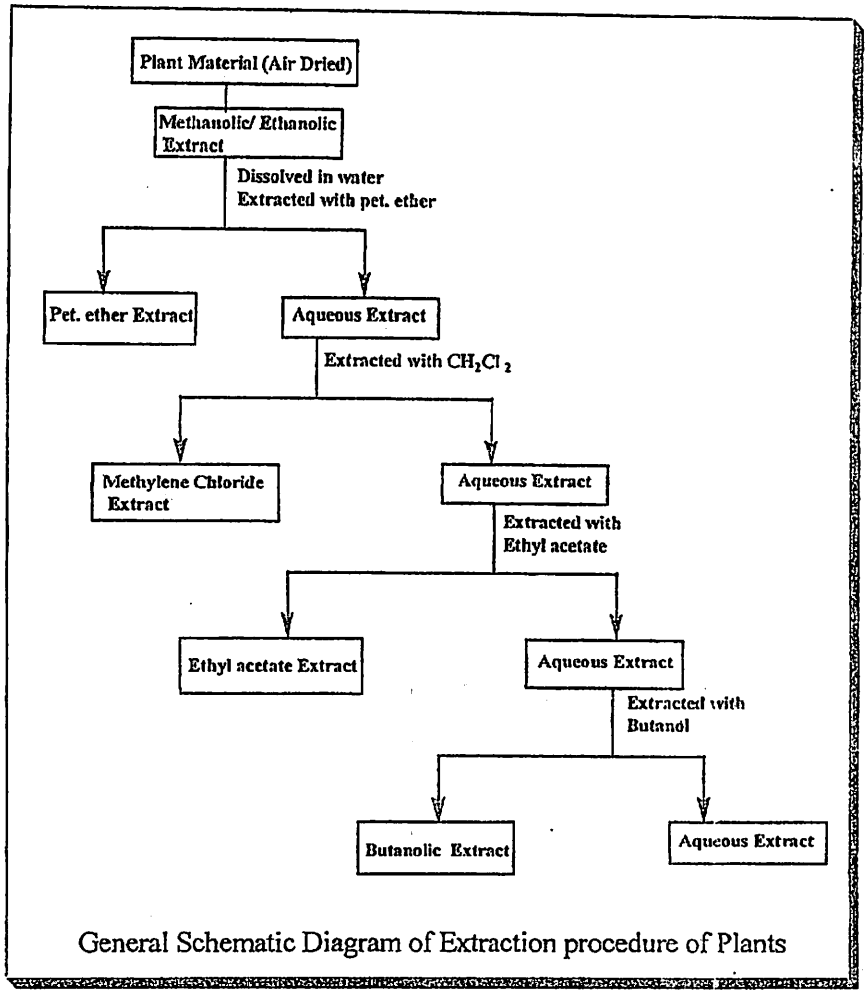
During this project a systematic study will be conducted on the chemistry and pharmacology of compounds isolated from natural sources and the library of the ICCBS compound bank. The main objective of this project is to evaluate the protective effects of natural products and their derivatives against pharmaceutically important enzymes: glucosidase, cholinesterase, and urease

Details of Proposed Isolation Work

Extraction and isolation of active constituents from medicinal plants will be carried out using a variety of chromatographic techniques, such as column chromatography, preparative thin layer chromatography, counter-current chromatography, and HPLC techniques. The structure elucidation of active constituents will be determined by using sophisticated spectroscopic techniques, and chemical methods.

Extraction and Isolation Plants will be collected (or purchased) and dried in air. Air-dried plant material will be crushed and socked in methanol/ethanol for 15-20 days at 25° C. The crude extract, obtained after the evaporation of the solvent, will be dissolved in water, and extracted with hexane to remove the non polar part. The aqueous extract will further be fractionated with $CHCl_3$, EtOAc and then with BuOH. These extracts will be evaporated and evaluated for their biological activities. Active extract will be fractionated and purified to pure compounds by using different chromatographic techniques like Silica gel column chromatography (CC), Sephadex LH-20 and HPLC. The column will be eluted with increasing polarities of different solvents like Hexane-CH_2Cl_2, Hexane-EtOAc, CH_2Cl_2-

EtOAc, CH_2Cl_2-MeOH, H_2O-MeOH, etc. to yield the most important active constituent form plant. The structure elucidation of active constituents will be carried out by using UV, IR, Mass, 1- and 2-D NMR techniques and chemical methods.



General Schematic Diagram of Extraction procedure of Plants

Different bioactive plant extracts yield pure compounds with inhibitory potential against the three pharmaceutically important enzymes: Glycosidase, Cholinesterase and Urease:

In vitro **Glycation Assay:** The assay will be perform in 96-well plate, each well will contain the reaction mixtures composed of BSA , glucose anhydrous and the test sample). Glycated control (BSA, glucose and Na_2PO_4 buffer) and blank control (BSA and Na_2PO_4 buffer) will also be maintained. The reaction mixture will be incubate at 37 °C for 7-days, followed by centrifugation for 4 minutes at 4° C after the addition of 100 % TCA in each well. The supernatant will be discarded and the solid will be dissolved PBS. The AGEs will be monitor by observing the fluorescence intensity change by using

Spectrofluorimeter and % inhibition will be calculated by the formula (Yamaguchi *et. al.*, 2000).

1 - [(Fluorescence of test sample) / (Fluorescence of glycated)] x 100

***In vitro* Cholinesterase Inhibition Assay**

The reaction mixture containing acetylcholinesterase (Ach) or butyrylcholinesterase (Bch), test compound, DTNB and PH 8.0 sodium phosphate buffer solution will be at incubated 25° C for 15 minutes. The yellow anoin (5-thio-2-nitrobenzoate) anion will be form due to the enzymatic hydrolysis of Ach or Bch and will be monitor at 412 nm by spectrophotometer for 15 min. All the reactions will be performed in and the percentage (%) inhibition will be obtained by using the following formula(Choudhary *et al.*, 2004) :

$(E - S)/ E \times 100$

E = Enzyme activity without sample S = Enzyme activity with sample

***In vitro* Urease Inhibition assay**

96-well plate containing jack bean urease and urea buffers and the test compound will be incubated for 15 min. Activity will be determined by indophenol method (Khan *et al.*, 2004) and the absorbance will be measured at 630 nm on a microplate reader followed by the calculation of the % inhibitions by using the formula $100-(OD_{testwell}/OD_{control}) \times 100$. Thiourea will be used as a standard inhibitor of urease in the assay (Khan *et al.*, 200).

4B. RESEARCH PLAN: SCHEDULE/PHASING (Not to exceed one page)

After the extraction, purification and *in vitro* screening of the compounds, the X-Ray Crystallographic technique will be used to study the precise and accurate three-dimensional structures of the target enzymes and the potent candidates which will serve as template for molecular-based drug designing for lead molecules. The structures will be modeled to interact with the active site, both by steric aspect and functional group interactions. Currently we have already several inhibitory compounds, identified for each of the target enzymes, as described in the scientific background section. Therefore, our scientists will determine the molecular structures of the target enzymes in complex with these inhibitory compounds; they will also apply X-ray crystallography to obtain the preliminary information regarding the binding of compounds to the target protein, and the changes associated due to these interactions. These information will be helpful to refine and improve the binding of the target proteins and the

optimization of lead molecules. The bioactive lead molecules from other studies, like screening of combinatorial libraries will also be optimized. Once the synthesis and optimization of the lead molecule is completed, it will be further evaluated for its activities in the physiological environment. The failure at any stage will lead to redesigning of the structural model by using the same strategy as described above, and repeated until the target molecule with desire properties is obtained.

The structural data obtained by the high-resolution single-crystal X-ray diffraction analysis will be the basis of designing of more effective enzyme inhibitors by employing conventional, computational, and synthetic medicinal chemistry methods. This partnership between the structural biologist, and medicinal chemists will play an important role in rational drug designing.

The project can be apportioned into four sections that are expounded in the following scheme:

- ✓ 1. Systematic screening of large libraries of natural products for their enzyme inhibitory properties.
- ✓ 2. Purification and crystallization of α-glucosidase, urease and cholinesterase with selected compounds.
- ✓ 3. Structural analysis of the enzyme-inhibitor complexes with synchrotron radiation at DESY/Hamburg.
- ✓ 4. Refinement and optimization of the lead compound to improve its binding to the target protein tests.
- ✓ 5. Evaluation of the optimized compound in a biological system and biochemical activity.
- ✓ 6. Virtual screening and development of novel pharmacophore models, based on the lead compounds.

	First Year						Second Year					
1	×	×	×					×	×	×		
2			×	×	×	×	×	×	×	×	×	×
3					×	×	×	×				
4							×	×	×	×	×	×
5					×	×	×	×				
6							×	×	×	×		

4C. REFERENCES (cited in 3, 4A & 4B; not to exceed two pages)

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5. IMPACT (of proposed research on teaching/training of manpower, institutional capability building and on local industry)

The three-dimensional structures of a drug target interacting with small ligands are used to facilitate the drug discovery. The use of single-crystal X-ray diffraction techniques by using information gained through crystallography, medicinal chemist redesign the structures of ligands to improve interactions with the active site. The redesigned lead compounds are synthesized and further checked again for their performance.

This project will set up a program to screen and identify known and novel natural products for their enzyme inhibitory activities. Such products will undergo further evaluation for their therapeutic potential. An attempt will be made to secure patents for promising compounds and their development into drugs via collaboration with national and international pharmaceutical companies using the HEJ and German links.

This project is collaborative in nature and will allow for exchange of knowledge and expertise in a new area of research between an established German laboratory and a leading Pakistani institution. The German group will have unrivalled access to plant material and natural products via the Pakistani group. Conversely, the Pakistani group will have access to expertise, training, literature and equipment at the German University. Pakistan has a history of using plant material for medicinal purposes and the HEJ Institute has isolated over 6000 natural products. However, there is a need to assess scientifically whether these natural compounds can be used to develop

marketable drugs with therapeutic potential. The current project investigates a novel area with little existing knowledge.

6. COLLABORATING LABS

In case of collaboration with national/international research group or local industry, please identify clearly the parts of research that will be carried out in the participating laboratories and please identify complimentarity and/or justify the need for collaboration) P.I.s are encouraged to find collaborating partners within Pakistan, particularly in less developed areas. Include a letter from Collaborating agency expressing willingness to collaborate.

1. **H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan**

a. Systematic screening of large libraries of natural products and their derivatives against various enzymes.
b. Study of the mechanism of action by using kinetic protocol.

2. **University of Hamburg, Institute of Biochemistry and Molecularbiology, c/o DESY Geb. 22a, Notkestraße 85, 22603 Hamburg, Germany**

a) Crystallization of selected complexes, X-ray diffraction data collection to high resolution

b) Structure analysis and refinement

c) Analysis of complexes and structure based design of optimized compound and inhibitors

The scientists involved in the proposed project have long term experience in the field of bioorganic chemistry and structural biology, the application and instrumentation of dynamic laser light scattering for crystallization experiments, structure analysis utilizing synchrotron radiation and in particular state of the art bioinformatics tools for virtual ligand screening. The X-ray application laboratory at DESY has therefore direct access to synchrotron radiation and benefits from the local instrumentation groups. Consequently also synchrotron radiation can be used, most flexible for all experiments and measurements. Therefore, the later on proposed working scheme can be followed most efficiently.

7. FACILITIES AND FUNDING

7A. Facilities: equipment available for the research project IN THE HOST UNIVERSITY/INSTITUTION

All instruments related to chromatographic techniques and sophisticated spectroscopic techniques for isolation and identification of active constituents from medicinal important plants are available in HEJRIC (list is given below).

We also have micro plate reader, with the help of which we can screen a large number of compounds at a time against a

variety of biological targets. This will save time, as well as application of this technology, need only few mgs of compounds.

1 Super-conducting Bruker Avance NMR spectrometers (300-600 MHz) including LC-NMR
2 High resolution Mass Spectrometers including Applied Biosystem LC-MS/MS, Thermofinigan MAT 95, Jeol HX-110 and JMS-600 mass range of over 12000.
3 LC-MS With ESI and APCI
4 State-of-the-Art, MALDI-TOF-TOF system (Ultraflex III Bruker)
5 Gas-Chromatograph/Mass Spectrophotometers (GC-MS)
6 Single Crystal X-ray diffractometers, P-IV and Apex Smart (Bruker)
7 ICP (Applied Biosystem)
8 FT-IR Spectrophotometers
9 HPLCs, including recycling HPLCs
10 UV Spectrophotometer
11 Abbe Benchtop Refractometers
12 Animal House Facility
13 Microbiological Unit
14 Cell/tissue Culture Laboratory
15 Patch clamp Facility for electrophysiology
16 ECT unit
17 Cryostat
18 Thermocycler
19 Single-Crystal X-ray Diffractometer
20 Gel-DOC system
21 Some basic chemicals such as adsorbents, media, and common reagents are available.

7B. Scientific Personnel

a. Available

b. Required*

Senior Research Associate (1)
Research Associate (2)

Lab. Attendant (1)

*Involvement of research students is encouraged.

7C. Other funding available for the proposed studies (if any)

The project will be supported by the German Institute via consumables with about Rs. 0.55 million / year in total

8. PRINCIPAL INVESTIGATOR

A brief resume of research accomplished in the last 05 years. Please specify title of the research proposal(s), duration, funding source(s) and award

amount(s).

CV Attached

8. PRINCIPAL INVESTIGATOR: continued

1.	Please attach C.V.	
2.	Number of Publications during the last five years & page numbers on the C.V. where these publications are listed	National:__ _____Please see pages_____ of CV International: 555 Please see pages : 5-45 of CV
3.	Number of research projects completed & page number where this information appears Applied: _____	Basic: _____ Please see pages _____ of CV Please see pages_____ of CV

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD

DESCRIPTION	% of time devoted to Project	YEAR 1 (Rs. in million)	YEAR 2 (Rs. in million)	Amount (in million Rs.)
A. Salaries and Honorarium				
PI: One month/year of basic salary @	10%	0.17	0.17	0.34
Co-PI:				NOT REQUIRED
Senior Research Associate (1) @ 25,000/ month		0.3	0.3	0.6
Research Associate (2) @ 13,000/ month		0.312	0.312	0.624
Typist		0.01	0.01	0.02
Accountant		0.01	0.01	0.02
Subtotal:		0.802	0.802	1.604

B. Permanent Equipment (Please attach invoice/quotation and expected delivery date for items costing over Rs. 0.1 million.)			
NOT REQUIRED			
Subtotal:			

C-1. Expendable supplies *(year wise quantity with full justification)*			
Material for cell cultures, HPLC, affinity-ion exchange and size exclusion columns	1.5	1.5	3.0
Plants/ Extracts, Chemicals / drugs / media, Solvents (For spectroscopy, HPLC, and chromatography)	1.0	1.0	2.0
Subtotal:	2.5	2.5	5.0

C-2. Training Cost (a total of three trainings for 6 months)			
	Two trainings (Rs. in million)	One training (Rs. in million)	(Rs. in million)
Travel cost / person (@ Rs. 0.08 million/ person)	0.16	0.08	0.24
Fellowship / month (@ Rs. 0.1 million/ person)	1.2	0.6	1.8
Subtotal:	1.36	0.68	2.04

9A. ESTIMATED BUDGET FOR THE PROPOSED RESEARCH PERIOD (continued)

DESCRIPTION	YEAR 1	YEAR 2	Amount (In million Rs.)
D. Others D1. Literature, documentation, information, online literature search, contingencies, postage, etc.			
i. Literature reprints			0.05
ii. contingencies, postage	0.01	0.01	0.02
Subtotal:			0.06

D2. Local Travel (Destination and purpose with full justification)			
For scientific meetings with the network partners, participation at workshops and symposia a total amount of is proposed	0.4	0.4	0.8
Subtotal:	0.4	0.4	0.8

D3. Miscellaneous			
Audit Fee (Max. Rs 10,000)			
Accountant Fee (Max. Rs. 10,000)			
Subtotal:			
Subtotal (D1 + D2 + D3):			
E. Indirect cost (University overheads) 02 % of Total direct cost to meet office support, utilities, etc.			
Grand Total (A + B + C + D+E):			9.504

9B. JUSTIFICATION (Please justify your request in a background of the existing facilities available at the host Institute.)

A. **Salaries & Allowances** (All positions, other than PI and Co-PI, must be fully justified. Please give qualifications/requirements of each of the new full-time positions requested for in the Proposal.)

Senior Research Associate: Ph. D. in Bioorganic Chemistry
Research Associate: M. Sc. (Ph. D. students)

B. **Permanent Equipment** (Please identify major items (over Rs. 25,000). Major pieces of equipment costing over Rs. 0.1 million must be fully justified. Minor items (under Rs. 25,000) may be lumped into one.)

No permanent equipment is required as most of the equipments are already available in collaborating institution.

C. **Expendable supplies** (With full justification and details of quantity required for the project)

Plant Material (5-8 Kg of dry weight of each) for phytochemical investigations

Solvents for extraction and purification of compounds
EtOH

MeOH
Hexane/Pet. Ether
CH_2Cl_2
EtOAc
BuOH

HPLC Grade solvents for the purification of natural products by HPLC method

MeOH
H_2O
Acetonitrile

Deutrated solvents for the NMR spectroscopic studies

Chemicals for Bioassays

BSA
Glucose anhydrous
Sodium phosphate buffer
Electric-eel acetylcholinesterase (EC 3.1.1.7),
Hourse-serum butyrylcholinesterase (EC 3.1.1.8),
Acetylthiocholine iodide,
Butyrylthiocholine chloride,
5,5'-dithiobis [2-nitrobenzoic acid] (DTNB)
Galanthamine
Jack bean urease
Urea
Phenol
Sodium nitroprussside)
NaOH
NaOCl)

All Chemicals will be purchased from Sigma or Aldrich.

D. **Other Costs.** (Travel must be justified.)

Travel for 6-8 months training (up to three trainings) of a Research Associate Officer and two Ph. D. students in Hamburg, Germany in the field of structural biology.

Project Proposal Case 03

PROFORMA PSF-I ®

SUBMISSION OF RESEARCH PROPOSAL FOR FINANCIAL ASSISTANCE TO PAKISTAN SCIENCE FOUNDATION

Name & Address of the Institution: HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

Title of Research Proposal: Studies on hepatoprotective effects of bioactive secondary metabolites of plants by using antioxidant and relevant bioassays

Main Field of Study: Bioorganic Chemistry

Nature of Research: (a) Basic ✓ Applied _____

* **Principal Investigator:**
(Attach Biodata)

** **Co-Principal Investigator:**
(Attach Biodata)

Proposed Duration: Two Years

Total Funds Requested: 1,249,200
(*Not more than 2.0 million*)

ENDORSEMENT:

Revised

1. Project Title:

Studies on hepatoprotective effects of bioactive secondary metabolites of plants using antioxidant and relevant bioassay

2. Project Abstract (Summary):

Liver is the main organ involved in the metabolism of biological toxins and medicinal agents. Such metabolism is always associated with the disturbance of hepatocyte biochemistry and generation of ROS (reactive oxygen species). Since the oxidative stress plays a pivotal role in the pathogenesis and progression of liver diseases, the use of antioxidants is proposed as therapeutic agents, as well as drug co-adjuvant, to counteract liver damage. We think antioxidants are able to reduce hepatic inflammation and fibrosis, thus slowing or even preventing the progression to cirrhosis.

Liver disorders pose a major challenge world-wide largely due to environmental and life style factors. Mortality and morbidity related to hepatic disorders is more in developing countries, while treatment is very often expensive and out of reach. In Pakistan, a large number of plants such as *Cassia tora, Andrographis paniculata, Acorus calamus, Phyllanthus niruri, P. amarus,* etc. are used for the treatment of liver disorders. Many of these plants have never been scientifically studied for their hepatoprotective effects. During this project we intend to carry out the first systematic study of selected medicinal plants of Pakistan for their hepatoprotective potential. A battery of *in vitro* and *in vivo* assay will be established to carry out these studies.

3. Project Narrative:

i) Significance of the proposed research duly supported with review of literature and bibliography to indicate current trends in the proposed field of study

ii) A brief account of work done in Pakistan/papers published thereof. and relationship of the proposed research to the socio-economic development of the country.

Liver is the main organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation. During the course of aerobic metabolic reactions, considerable amounts of Reactive Oxygen Species (ROS) such as superoxide anion (O^{-2}) and hydrogen peroxide (H_2O_2) are generated, which undergo a variety of chain reactions and produce free radicals such as OH°. These hydrogen species attack the polyunsaturated fatty acids and thereby initiate the process of lipid peroxidation, resulting in degradation and inactivation of various important biomolecules [1].

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved an extremely efficient and highly sophisticated protective system, the so-called "antioxidant defensive system". It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals [2]. A broader definition of an antioxidant is "any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those substrates". To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects [3].

Every antioxidant has some significance and the best protection against oxidative stress comes from the presence of a wide assortment of interrelated antioxidants and their cofactors. The function of each particular antioxidant depends on what type of oxidative stress is imposed [2].

Lipid peroxidation can damage low-density lipoprotein (LDL) particles in several ways. *In vitro* studies have demonstrated that lipoxygenase, superoxide anion, peroxynitrite, and myeloperoxidase can oxidize LDL, which can lead to heart diseases. Studies showed that antioxidants may protect against coronary heart diseases [4-7]. Vitamins have been shown to reduce the susceptibility of LDL to oxidation and are also known to be involved in elevating the level of protection factors like HDL-cholesterol. Studies suggest that vitamin C may reduce the risk of hypertension [8]. In addition, a high intake of vitamin C appears to protect against gastric cancer, probably through scavenging ROS formed in gastric mucosa [9]. Further investigations on vitamin C have proved its preventive effects on inhibition of tumor promotion [10].

Dietary antioxidants are important to maintain good health. Vegetables and fruits contain a variety of important nutrients that are essential for the normal functioning of the body. Tomato intake, the main source of lycopene, has been found to be associated with a lower risk of a variety of cancers in several epidemiological studies. It was reported that supplementations with vitamins C and E, mixed with other antioxidants, can reduce symptoms of oxidative stress during exercise.

Flavonoids are polyphenols which are abundantly found in fruits, vegetables, grains, bark, roots, stems, flowers, and tea. They possess anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties due to their antioxidant potential.

Carbon tetrachloride is one of the most commonly used hepatotoxins used in the experimental study of liver diseases. It was found that chronic administration of CCl_4

produced liver cirrhosis in rats. Carbon tetrachloride is biotransformed under the action of cytochrome p 450-2el (CYP2e11) in the microsomal compartment of liver to trichloromethyl ($^{\circ}CCl_3$) and peroxytrichloromethyl (CCl_3COO°) free radicals. These free radicals bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides followed by pathological changes such as:

- Triglyceryl accumulation due to blockage in synthesis of lipoprotein
- Polyribosomal disaggregation
- Depression of protein synthesis
- Elevated levels of serum marker enzyme such as SGOT, SGPT, and ALP
- Depletion of glutathione
- Increased lipid per oxidation
- Cell membran break down and death

The elevated level of serum enzymes are indicative of cellular leakages and loss of functional integrity of cell membrane in liver, serum ALP and bilirubin levels on the other hand are related to the function of hepatic cells. CCl_4 intoxication also produces significant rise in serum bilirubin thereby indicating hepatic damage.

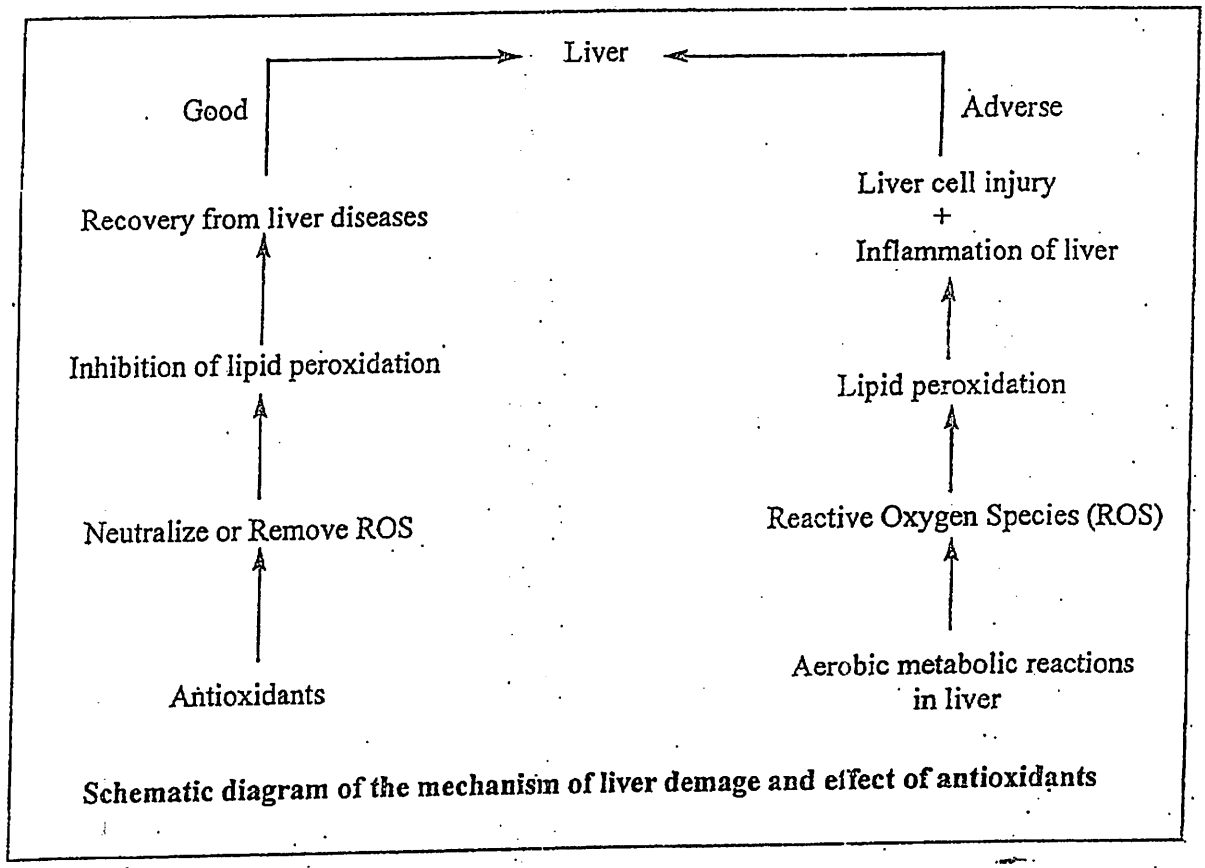
Lots of liver disorders ranging from subclinical icteric hepatitis to necroinflammatory hepatitis, cirrhosis, and carcinoma have been proved to associate with the redox imbalance and OS (oxidative stress) [11]. Therefore, there is need to develop antioxidants drugs to treat and protect liver injury and liver diseases.

This strategy is aimed to devise and incorporate antioxidants into the therapeutic for control of viral infections or protecting body from alcohol or other toxin damage. We think antioxidants are able to reduce hepatic inflammation and fibrosis, thus slowing or even preventing progression to cirrhosis.

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases including liver diseases, ischemia, reperfusion injury, atherosclerosis, acute hypertension, hemorrhagic shock, diabetes mellitus and cancer.

The present study was designed to induce CCl_4 hepatotoxicity in experimental animal models and demonstrate the protective role of natural products and plants extracts trough their

antioxidant effects. This will lead to the identification of effective antioxidant plant materials with proven liver protective effects.



Schematic diagram of the mechanism of liver demage and effect of antioxidants

Recent Literature Review:

In literature hepatoprotective activities of several medicinal plants *Ficus bengalensis* [12], *Zedoariae Rhizoma* [13], *Mentha arvensis, Sophora japonica, Benincasa hispida, Lonicera japonica* (Lonicerae Flos), *Agaricus blazei, Epimedium koreanum, Aralia continentalis, Lithospermum erythrorhizon, Cimicifuga foetida, Gastrodia elata, Sanguisorba officinalis, Cephalonoplos segetum, Bupleurum falcatum, Alisma plantago-aquatica var. orientale, Lonicera japonica* (Lonicerae Folium), *Sinomenium acutum* [14] and *Phyllanthus niruri* [15] and other herbs [16], were evaluated and aim was to develop hepatoprotective drugs.

In Pakistan, Gilani and Jahanzeb studied the hepatoprotective activity of aqueous-methanolic extract of *Cyperus scariosus* (Cyperaceae) against the acetaminophen and CCl_4-induced hepatic damage [17].

References:

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iii) Specific Objectives and expected results.

The aim of this project is to identify natural chemicals derived from plant sources in Pakistan that can be utilized as hepatoprotective agents. The objectives are:

- To identify medicinal plants and pure phytochemicals with antioxidant potential as a approach towards the treatment and management of liver associated diseases.
- To screen (*in vitro*) selected natural products and plants extracts for their ability as an antioxidants.
- To investigate (*in vivo* in appropriate experimental animal model) whether these natural products or their constituents can protect liver against the harmful effect of CCl_4 induced toxicity.
- To study the mechanism and site of action of promising compounds.
- To derivatize most promising natural products and study their effects as hepatoprotective agents.

iv) Description of research methodology/techniques to be used including critical or difficult phases or factors and how these will be investigated.

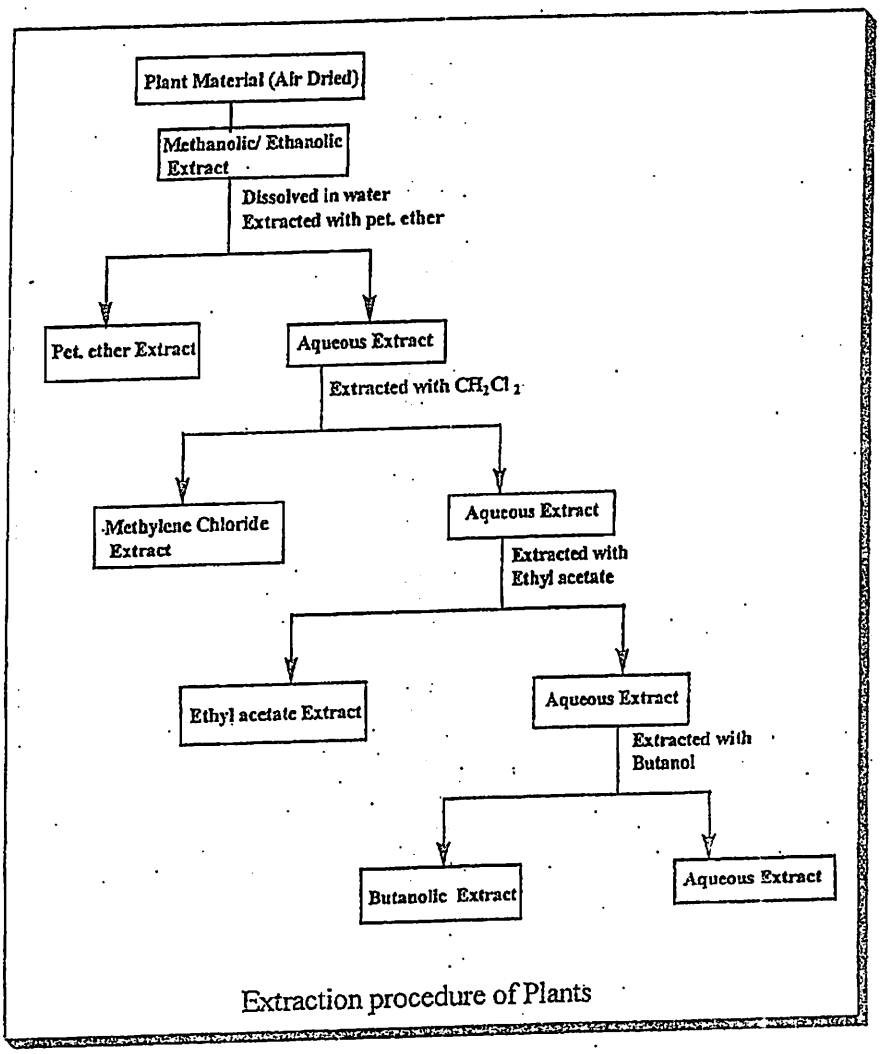
During this project a systematic study will be conducted on the chemistry and pharmacology of compounds isolated from natural sources. The main objective of this project is to evaluate the protective effects of natural products and their derivatives against hepatotoxicity in mice produced by CCl_4 and some other drug and to investigate possible mechanism/s underlying these effects. Protection will be assessed by monitoring liver function/dysfunction.

Extraction, and isolation of active constituents from plants will be carried out using a variety of chromatographic techniques such as column chromatography, preparative thin layer chromatography, counter-current chromatography and HPLC techniques. The structure elucidation of active constituents will be determined using sophisticated spectroscopic techniques and chemical methods.

Details of Proposed Work

Extraction and Isolation

Plants will be collected (or purchased) and dried in air. Air-dried plant material will be crushed and keep in methanol/ethanol for 15-20 days at 25° C. After evaporation of the solvent, a crude extract will be obtained which will be dissolved in distilled water and defatted with hexane. The defatted aqueous extract will further be fractionated with $CHCl_3$, EtOAc and then with BuOH. These extracts will be evaporated and evaluated for their antioxidant activity. Active extract will be subjected to column chromatography (CC) on Silicagel, Sephadex LH-20 and HPLC and eluted with gradients of different solvents like Hexane-CH_2Cl_2, Hexane-EtOAc, CH_2Cl_2-EtOAc, CH_2Cl_2-MeOH, H_2O-MeOH etc. to yield the most important active constituent form plant. The structure elucidation of active constituents will be carried out by using UV, IR, Mass, 1- and 2-D NMR techniques and chemical methods.



Extraction procedure of Plants

Proposed Plants to be Studied

The major goal of this project proposal is to isolate and identify the active constituents from medicinal plants, which were used traditionally by indigenous population for the treatment of the liver disorders. The plants which have selected for the study on the basis of ethanobtanic survey are as follow

1 *Picrorhiza kurroa*
2 *Cucuruma longa*
3 *Camellia sinensis*
4 *Allium sativum*
5 *Acorus calamus*
6 *Andrographis paniculata*
7 *Phyllanthus niruri*
8 *P. amara*
9 *Cassia tora*
10 *Plumbago zeylanica*
11 *Tinospora cordifolia*
12 *Swertia chirayita*
13 *Azadirachta indica*
14 *Cuscuta reflexa*
15 *Boerhavia diffusa*
16 *Chelidonium majus*
17 *Cichorium intybus*
18 *Eclipta prostrate*
19 *Glycyrrhiza glabra*
20 *Withania somnifera*
21 *Withania coagulance*
22 *Ficus virgata*
23 *Peganum harmala*
24 *Mentha piperita*

Following assays will be used for measuring the antioxidant potential of the test samples.

1. **DPPH Radical Scavenging Assay:**

Free radical scavenging abilities of the test compounds could be determined by measuring the change in absorbance of DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) at 515 nm by the spectrophotometric method .

2. **Superoxide Anion Scavenging Assay:**

Superoxide scavenging activities of compounds could be determined by using the method described by N. S. C. Gaulejac (R). The assay involves a non-enzymatic generation of superoxide anions ($O_2^{\bullet-}$). The superoxide anion scavenging activity could be determined by measuring the reduction in rate of formation of formazan dye.

3. **Xanthine oxidase inhibition assay:**

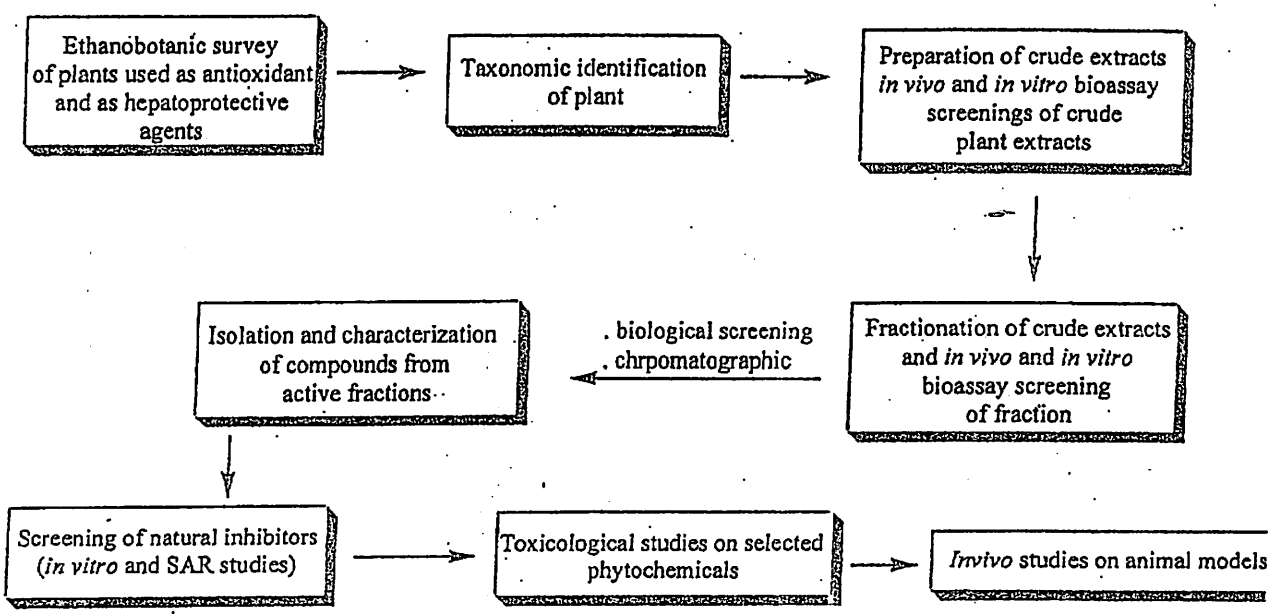
Xanthine oxidase is a cytosolic molybdenum containing iron sulfur flavoprotein, which catalyzes the oxidative hydroxylation of a broad range of aldehydes and aromatic heterocyclics. Under certain conditions, xanthine oxidase can be a major source of $O_2^{\bullet-}$ intracellular production. The enzyme activity could be determined by measuring the rate of hydroxylation of the substrate (xanthine) with the formation of uric acid, which is a colorless end product of the reaction and shows absorption at 295 nm.

***In vivo* Hepatoprotective Studies:**

The *in vivo* hepatoprotective activity of selected compounds will be studied by CCl_4 induced hepatotoxicity in rat models. Carbon tetrachloride is a toxic molecule and it is used to produce liver damage. CCl_4 will be converted to $^{\bullet}CCl_3$ and $CCl_3COO^{\bullet}$ which may attack cellular membranes which finally results in the rupture of the structure of bio-membranes, resulting in large quantities of cytosolic enzymes released into the blood stream.
The normal control will be received using saline solution whereas the pathological control will be given an i.p. dose of CCl_4. The effect of saline solution and CCl_4 will be monitored by the estimation of serum enzymes (AST, ALT) and bilirubins using standard Kit method.
The effects of natural and synthetic compounds will be studies by pretreatment of the animals with different doses. The results of the activities by different compounds will be compared with pathological control group.

Cytotoxicity Studies:

The natural and synthetic compounds, which were found to be hepatoprotective, will be subjected to cytotoxicity assay. The cytotoxic activity of compounds was studied by using human neutrophils. An *in vitro* spectrophotometric method could be used for this study which measures the cell viability (%) after the incubation of test compounds with human neutrophils. The assay is based on the reduction of tetrazolium salt WST-1 by mitochondrial dehydrogenases of viable cells to yellow organ formation dye, which can be measured spectrophotometrically.



iv) Year-wise plan of work.

The project can be apportioned into four sections that are expounded in the following scheme:

- ✓ 1. Collection of data/ purchase of chemicals
- ✓ 2. Collection/Purchase of plants/extract preparation
- ✓ 3. Phytochemical studies and derivatization of selected compounds.
- ✓ 4. Systematic *in vitro* screening of large libraries of natural products for their antioxidant properties.
- ✓ 5. Studies of toxicological profile of potent inhibitors

✓ 6. Establishment of *in vivo* on animal model and evaluation of potent antioxidants for hepatoprotective activity.

✓ 7. Publication / patenting of the results.

	First Year						Second Year					
1	×	×					×	×				
2	×	×	×				×	×	×			
3		×	×	×	×	×	×	×				
4			×	×	×	×	×	×	×			
5					×	×	×	×				
6					×	×	×	×	×	×	×	×
7										×	×	×

v) Expected benefits of the proposed study.

Liver associated diseases are one of the huge health problem in Pakistan, particularly in disadvantaged communities, because of their poor access to modern healthcare facilities. Many individuals therefore rely on non-traditional medication such as plant extracts. This project will set up a programme to screen and identify existing and novel natural products for their hepatoprotective properties. Such products will undergo further evaluation for their therapeutic potential and may protect against its complications. An attempt will be made to secure patents for promising compounds and their development into drugs via collaboration with national and international pharmaceutical companies.

4. Scientific personnel required for the project.

Research Associate (1)

5. Existing institutional facilities.

All instruments related to chromatographic techniques and sophisticated spectroscopic techniques for isolation and identification of active constituents from medicinal important plants are available in HEJRIC.

We also have micro plate reader, with the help of which we can screen a large number of compounds at a time against a variety of biological targets. This will save time, as well as application of this technology need only few mgs of compounds.

The institute has animal resource facility to facilitate animal use in research at the ICCBS, University of Karachi by providing high quality animal care in accordance with Institutional regulations and guidelines. This facility will support *in vivo* studies of the project.

6. **State if the scheme has been submitted to some other aid giving agency for financial support. If so, with what results?**

The project has not been submitted to any funding agency

7. **Patentability of the project results.**

There is enough potential that most active constituents of plants could be patented for its hepatoprotective activity.

8. End user (s) of the project results in case of applied research project (attach letter (s) of intent from the end user (s).

9. Indicate, as per details given below, other research projects being conducted or previously guided by the Principal and Co-Principal Investigator, if any.

Project Title	Duration	Total Amount of Grant	Funding Agency
CV Attached			
I)			
Ii)			
Iii)			
Iv)			
v)			

PROPOSED REVIEWERS OF THE PROJECT

Names and addresses of three reviewer's/referees having sufficient research experience relevant to the proposed study may be provided on a separate sheet of paper.

10. **Research Proposal Budget:**

TABLE-I

Estimated Cost of the Project in Rupees (Table-II to V)

Year:	Recurring (Salary/honorarium and allowances)	Non-Recurring (Equipment and Res. Materials)	Total
Ist Year:	160,600	464,000	651,000
2nd Year:	160,600	464,000	651,000
Grand Total:	321,200	928,000	1,249,200

TABLE-II

Expenditure on Salaries and Allowances

Post & Scale of Pay	No. of Posts	Ist Year	2nd Year	Total
Honoraria -Principal Investigator (@ Rs. 35,000/- per year)	1	35,000	35,000	70,000
-Co-Principal Investigator (@ Rs. 20,000/- per year)	1	20,000	20,000	40,000
(II) **Allowances:** -Professional/Technical Personnel (Research Associate) @ Rs.8,000/- per month	1	96,000	96,000	192,000
-Other Personnel Typist/ Accountant @ 800/month	1	9,600	9,600	19,200
(III) **Travel within country:** (For projects involving field work only)	Not Required			
Total:-		1,60,600	1,60,600	3,21,200

Note: Please give full justification for the staff as well as for travel within country.

TABLE-III

Expenditure on Equipment & Supplies

Sr.No.	Item	Ist	2nd	Total
1.	Permanent Equipment	Not required		
2.	Plant extracts, animals, chemicals, drugs, media, solvents for chromatography, HPLC and spectroscopy	350000	350000	700000
3.	Glass-ware, disposable items,	100000	100000	200000
4.	Stationery	6000	6000	12000
5.	Literature	3000	3000	6000
6.	Contingencies, Postage etc.	5000	5000	10000
7.	Any other (Please specify)	-	-	-
Total:		464000	464000	928000

TABLE-IV

Receipts, if any from other sources for this Proposal

	Sources		
Year	(a)	(b)	(c)
Ist Year:	No funding available		
2nd Year:	No funding available		
3rd Year:	No funding available		
Total:			

TABLE-V

List of Permanent Equipment Required for Proposed Work

Sr.No.	Item:	Quantity	Approx. Cost
	Not required		
Total:			

Note: Please give:

i) **Justification for each item of permanent equipment(s).**
ii) **Specifications of equipment.**
iii) **Equipment cost be supported by proforma invoices from Principal Suppliers.**