

PHARMACOGNOSTIC AND
PHYTOCHEMICAL STUDIES OF
HEDERA HELIX L.
(*HEDERA NEPALENSIS* K. KOCH)

BY
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DEPARTMENT OF BOTANY
UNIVERSITY OF PESHAWAR
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DEDICATION

***THIS THESIS IS DEDICATED TO MY CHILDREN AND
STUDENTS.***

PHARMACOGNOSTIC AND PHYTOCHEMICAL STUDIES
OF
HEDERA HELIX L. (*HEDERA NEPALENSIS* K.KOCH)

BY
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A Thesis
submitted to the University of Peshawar in partial
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ABSTRACT

Hedera helix L. is an important medicinal plant found in the forests of N-W.F.P., as an ever green climber.

The pharmacognostic and phytochemical studies carried out on *Hedera helix* leaves showed that it is heterophyllus, but histologically the leaves are similar. The palisade parenchyma is multi - layered, stomata are present only on the lower epidermis. The palisade ratio is 1.4, stomatal index is 10.8 and vein islet No. is 12.4. The spongy parenchyma contain large rosette aggregates of Calcium oxalate.

Antidiabetic studies carried out on both healthy and alloxan induced diabetic rabbits showed that *Hedera helix* leaves possess antidiabetic activity . Of the two extracts used, the aqueous extract was more active than the methanolic one. The reduction in blood glucose level is more significant at 4-12 hours interval. Phytochemical investigations revealed that *Hedera helix*

leaves contain significant quantities of Cr, Zn and Mn, all of which have important physiological role in glucose metabolism.

The percent yield of the saponinic content of the leaves of *Hedera helix* collected from different climates showed no significant difference indicating that the saponin synthesis is a genetically controlled process and least affected by the climatic variations.

Cytotoxic studies, using Brineshrimp bioassay showed that *Hedera helix* leaves possess cytotoxicity. Subsequent phytochemical investigation indicated that the cytotoxic principle of *Hedera helix* leaf is a phenolic compound.

A preliminary screening test against some local pathogenic bacteria confirmed the antibacterial activity of *Hedera helix* saponins.

CHAPTER-I

INTRODUCTION

CHAPTER-I

INTRODUCTION

Mankind started the treatment of his ailments with plants and animals on a "hit and trail basis" and his knowledge passed to the successive generations with further additions at each generation. From that earlier era, the art of healing passed through various stages (Tyler, 1981). The use of medicinal plants in the treatment of ailment is of remote antiquity. Ancient nations, the Assyrians, the Babylonians, the Greeks, the Chinese, the Indians, the Persians and especially the Egyptians were greatly interested in medicinal herbs and profoundly believed in their healing effects. Egyptians considered medicinal herbs sacred and made from them gods to be worshipped.

Knowledge of medicinal plants has its origin in the fifth and fourth centuries B.C. under the patronage of Hippocrates in Greece and from there this system moved to

Rome, Alexandria, Byzantium, and finally to Jundishapur, a town in Iran, and in the middle of eight century it came to Baghdad, and from 750 A.D. to 850 A.D., learned Muslim, Christian and others scholars translated a large number of important works of Philosophy, Tibb, and different sciences into Arabic. The 'CANNON' of Avicenna remained the MEDICAL BIBLE of the whole of Europe for many centuries (Said, 1991).

The history of medicine in India can be traced to the remote past. The earlier mention of the medicinal use of plants is to be found in the 'Rigveda' which is one of the oldest, if not the oldest, repositories of human knowledge, having been written between 4500 and 1600 B.C. (Chopra et al., 1956)

In the " Atharvaveda " , which is a later production, the use of drug is more varied, although it takes the form, in many instances of Charms, Amulets, etc. It is in the " Ayurveda ", which is considered as an " Upaveda " (or supplementary hymns designated for the more detailed

instruction of the mankind) that definite properties of drugs and their uses have been given in some detail. "Ayurveda" in fact, is the very foundation stone of the ancient medical science of India. It has eight divisions, which deal with different aspects of the science of life and the art of healing. The age of Ayurveda is fixed by various Western Scholars somewhat about 2500 to 600 B.C. (Chopra et al., 1956)

During the time of Great Ashoka, the Hindu materia medica contained about 700 vegetable drugs which were used by the Vaidyas. They were mostly cultivated in gardens all over the country.

As the science progressed, the whole crude drugs were replaced by their extracts and galanicals. In the modern era, the chemical nature of the active constituents in crude drugs, their isolation, purification and their synthesis has tremendously advanced the medical science. Most of the recent medicines are either synthetic or prototypes of the natural drugs, which, though very

effective, are very costly and have many serious side effects and other consequences. These facts along with present socio-economic and industrial factors, convinced the mankind to revert back to "nature". A greater population of the underdeveloped and developing nations are still using natural folk medicines. The developed West, which until recent past has almost abandoned the crude drugs, is again recognizing the usefulness and advantages of those natural remedies. Recent analysis in the USA show that 20% prescriptions at community pharmacies contain substances derived from higher plants (Tyler, 1986). Plant drugs are less toxic and free from side effects as compared to synthetic chemicals with similar pharmacological effects (Momin, 1987).

Like heart diseases, hypertension and cancer, the disease of "Diabetes mellitus" has also increased many fold in recent years due to so called human progress and developments of the modern age.

There are large number of plants used by local people of the subcontinent for the treatment of hypoglycemia (Nadkarni, 1954 ; Campbell, 1958). Plants contain different physiologically active compounds, e.g. Alkaloids (morphine, cocaine, atropine etc.), Glycosides (Saponin glycosides, Cardioactive glycosides and anthraquinone glycosides etc.), Volatile oils (Peppermint, clove, cardamom oil etc.), resins, gums and mucilages. All these compounds are present in different ratios alone or in combination with other compounds and have different physiological activity when used in combined form or alone.

Plants have been used as source of drug administered empirically or otherwise in cure of disease for centuries. A large number of indigenous medicinal plants have been used to treat various ailments of mankind and animals since centuries by the practitioner of indigenous systems of medicine either singly or in combination with other plants. Several such plants have been screened by the modern pharmacological research methods and have been

found to possess physiologically active principles useful for treating various diseases (Lewis and Elins-Lewis, 1977).

It has been reported that indeed today many, if not most, pharmacological classes of drugs include a natural product prototype (Gilani, 1992). Morphine, atropine, reserpine, quinine and digoxin are the few example of drugs of plant origin still used in modern day therapeutics. Though a very large number of plants have been investigated and used for their medicinal properties, yet the list of plants that have not been studied for their physiologically active constituents is very long. Only 1-2% of terrestrial plants have been investigated properly (Farnsworth, 1983).

As herbal drugs are considered to be less toxic and more free from side effects than synthetically developed drugs so today, there is a great potential for the industrial development of traditional herbal remedies and

plant extracts for the treatment and prevention of disease (Bannerman, 1983).

The medicinal value of many plants previously being employed empirically against diabetes mellitus, skin and other diseases, has now been confirmed (Bahjiri et al., 1984). *Catharanthus roseus* (*Vinca rosea*), for example, is prescribed as an oral antidiabetic agent in the folk medicine and its galenical preparations are also being marketed in certain countries under the proprietary names of "Covnica" and "Vinculin". Recent research has now confirmed that this plant contains many alkaloids and some of its alkaloids like vindoline and leurosine have been reported to possess high degree of hypoglycemic properties (Lewis and Elvin-Lewis, 1977). Similarly two other alkaloids of *Catharanthus roseus*, Vinblastine and Vincristine possess anticancer activity and are available as prescription drugs for the treatment of generalized Hodgkin's disease, choriocarcinoma and acute leukemia (Tyler et al., 1981). Onion (*Allium cepa*) and garlic

(*Allium sativum*) have also been used as antidiabetics (Lewis and Elvin-Lewis, 1977). This fact was confirmed by Akhtar et al. (1981 and 1982) by their findings of potent hypoglycemic principles like allyl propyl disulphide (APDS) and allicin. Later on Akhtar et al. (1986) have shown that *Momordica charantia* fruit exerted a strong and consistent hypoglycemic effect in rabbits and man due to its active alkaloidal constituent "Charantine". Baldwa et al. (1977) have isolated an insulin like compound from fruits as well as tissue cultures of *Momordica charantia* and showed that the substance possesses a consistent hypoglycemic effect in patients with diabetes mellitus. Similarly Akhtar (1992) has reviewed twenty six indigenous plants and has described several, e.g. *Fumaria parviflora*, *Euphorbia prostrata*, *Cuminum nigrum* etc., to possess hypoglycemic properties.

Hedera helix (Araliaceae) is potentially a very important medicinal plant. The active constituent of this plant are physiologically active with antibacterial,

antifungal and cytotoxic properties (Quetin-Leclercq et al., 1992). It is reputed as an antidiabetic in some parts of the North-West Frontier Province of Pakistan. An oncologist of Peshawar Dr. Ilyas has informed (in writing) that one of his patients, suffering from advanced stages of Hodgkin's lymphoma recovered from this disease, when he used *Hedera helix* water extract. Due to the alleged antidiabetic and anticancer (in Hodgkin's lymphoma) properties, *Hedera helix* has become an important potential medicinal plant of this region.

Due to a variety of reasons the number of diabetic patients are increasing all over the world. This disease is almost incurable. The only remedy is to control it through diet and medicines. The present available medicines for the control of diabetes are either expensive or have several side effects. If *Hedera helix* proved effective as a hypoglycemic agent, it would be another great contribution of plant kingdom in the fight against one of the serious diseases of mankind. As *Hedera helix* is abundantly available and can also be easily propagated in

temperate forests of N-W.F.P, it can be exploited on commercial basis as a cost effective remedy against diabetes.

At present due to environmental pollution, smoking and excessive use of different chemicals in foods, cosmetics and toiletries, the risks of malignant cancerous diseases are high. The available chemotherapeutic agents for cancerous diseases are not only very expensive, but also have very serious side effects. For example, mustard hydrochloride, uracil, carmustine (BGN₄), decarbazine (DTK), vinblastine and vincristine-sulphate, etc. All these drugs cause serious nausea, vomiting, bone marrow depression, lymphocytopenia, granulocytopenia, thrombocytopenia with constant hemorrhage, vertigo, deafness. Carmustine (BGN₄) causes renal and hepatic damages along with bone marrow depression and pulmonary fibrosis. Vinblastine and vincristine are obtained from *Catharanthus roseus* but the yield is very low, as 500 kg of the plant material produce only 1.00 gm of vincristine which makes these drugs very expensive. Moreover, these

vinca alkaloidal drugs cause leucopenia, headache, depression, psychosis, constipation, convulsions and have also damaging effect on lungs, heart, kidneys and reproductive systems. Most of these anticancer drugs also cause alopecia (baldheadedness) and damage eye sight. A preliminary anticancer (cytotoxic) study of *Hedera helix* plant with positive result will open a vast field for further research work.

Due to the frequent prescription and consequent use of second and third generation antibiotics by patients, the resistance to the available antibiotics is increasing and the search for new and more effective antibiotics/antibacterial agents is continuously going on. The antibacterial and antifungal activity of *Hedera helix* against large number of organisms has been established (Cioaca et al., 1978). A further screening for the antibacterial activity against some other pathogens would further increase the list of pathogens, susceptible to *Hedera helix*.

1.2 OBJECTIVES

The present work is aimed to undertake the pharmacognostic and some phytochemical investigations of *Hedera helix*.

1.2.1 Pharmacognostic Studies

Before proceeding to phytochemical studies on *Hedera helix* plant, it is important to undertake pharmacognostic studies of this plant. Which would include identification, proper collection, drying, morphological, histological and other diagnostic features of *Hedera helix*.

1.2.2 Antidiabetic (Hypoglycemic) Study of *Hedera helix*

Antidiabetic study of *Hedera helix* would be carried out to confirm its reputed hypoglycemic activity, as used in the folk medicine.

Some of the trace elements, e.g. Chromium (Cr), Zinc (Zn) and Manganese (Mn) have vital role in the

carbohydrate metabolism (Amjad et al., 1993). Therefore it is also planned to evaluate *Hedera helix* for its Chromium, Zinc and Manganese contents.

1.2.3 Phytochemical and Cytotoxic Studies of *Hedera helix*

Brineshrimp Bioassay (Meyer et al., 1982) would be carried out to find out the cytotoxic effect of *Hedera helix*. Any substance which is cytotoxic is potentially an anticarcinogenic and can be proved anticarcinogenic. The data obtained would be processed using computer Fenney Programme to find out LC_{50} .

For the determination of chemical nature of the compound, Ultraviolet and Infrared spectroscopies and chemical tests (for confirmation of the chemical nature) will be performed

1.2.4 Antibacterial Activity of *Hedera helix*

Antibacterial screening bioassay of some available pathogenic bacteria would be carried out at some

institution, having the required microbiology
laboratory.

1.3 REVIEW OF LITERATURE

Mahran et al. (1974) found that the main constituents of *Hedera helix* were α -hederin, hedrasaponin B and hederasaponin C. The plant also contains unsaturated sterols, tannins, nitrogenous bases, oxides and flavonoids.

Timen-David et al. (1980) worked on the antifungal activity of the extract of *Hedera helix*. They extracted various saponins. These saponins were then tested for their antifungal activities both *in vitro* and *in vivo* in mice infected by *Candida albicans*. All the four saponins tested were able to control the disease.

Brandet (1967) prepared an antitussive, antispasmodic and anti-inflammatory extract from *Hedera helix*.

Pant (1988) extracted three types of compounds and named them as Nepal-I, Nepal-II and Nepal-III. He tested the spermicidal activity of these saponins in human

being and found them very effective in immobilizing the human sperms at 0.5, 0.25 and 0.125 percent concentrations respectively.

Marlier (1987) extracted from the leaves a yellow oil (1.2%). The major components in the oil were petroselinic acid (58.7%), oleic and vaccenic acid (11.3%), palmitoleic acid (11.3%) and linoleic acid (10.5%).

Kizu et al. (1985) isolated twelve saponins from *H. nepalensis* K. kech (*Hedera helix* L) and named them N-H saponins, which included A(I), B(II), D(III), D, (IV), F(VI), H(VII), I(VIII), K(X), M(XII), N(XIII) and P(XIV).

Jules (1964) isolated six different saponins from *Hedera helix* and observed that some of the saponin possess antimitotic power and inhibit the growth of cells without destroying them.

Lukic et al. (1975) extracted fruit of *Hedera helix* with pet. ether and characterized the oil obtained. The six fatty acids found and their percent compositions were

myristic acid (0.10%), palmitic acid (5.25%), Palmatoleic acid (1.72%), Stearic acid (0.70%), oleic acid (88.12%) and Linoleic acid (4.2%).

Mahran et al. (1974) analyzed the nature of linkage in the isolated glycosides (saponins) of *Hedera helix*. They reported that *Hedera helix* contain α -hederin (I), hederasaponin B(II) and hederasaponin C(III), I & III contained the aglycon hederagenin, whereas II contained oleanolic acid. I contained arabinose and rhamnose, attached by β -linkage to the 3-OH of hederagenin but with an d-linkage between them. II and III contained 1:2:2 arabinose - rhamnose glucose connected with a p-linkage at the 3-OH, the C-17 carboxyl group was free.

Mahran et al. (1975) isolated an alkaloid "emetine" from the alcoholic extract of the four varieties of *Hedera helix* growing in Egypt.

Jules et al. (1964) has described a method for large scale extraction of saponins from *Hedera helix* leaves and their use in inhibiting and stopping the growth of malignant tumors and for combating protozoa in particular trichonomas.

Maffei Facino and Carni (1990) reported that topically applied saponins, extracted from *Hedera helix* leaves cured in 30 days, 90% of the patients of liposclerosis (skin roughness, skin elasticity, sensitivity to provoked pain, skin toughness, consistency of the subcutaneous tissue and lipo-oedema) to an extent comparable to that produced by the well known anti-aedematous agent "escin".

Elias et al. (1990) tested the antimutagenic activity of *Hedera helix* along with 2 Spp of calendula. They have reported the antimutagenic activity of saponins of *Hedera helix* against a well known promutagen, Benzo-(a) pyrone

(Bap) (1 μ g) and a mutagenic urine concentrate from a smoker (SU) (5 μ g).

Hiller (1987) has reported the carcinostatic activity of some saponins.

Agarwal and Rastogi (1974) have reported significant antimitotic activity of glycosides (saponin) and their magnesium salts.

Majester Savornin et al. (1991) worked on the Antileishmanial activity of the saponins of *Hedera helix* and showed that these saponins were as effective as the reference leishmanicidal compound (Pentamidine).

Bosshard (1992) tested extracts of *Hedera helix* leaves, and showed that these extracts inhibited the conidial germination of *Vinturia inaequalis* and showed a preventive activity against scab and powdery mildew on apple seedlings under controlled conditions.

Cioaca et al. (1978) tested the antibacterial activity of the saponins from *Hedera helix* against 29 bacteria and one yeast genus and showed that the saponin was bactericidal against all the 23 tested strains. He reported that the saponins were generally more active against gram-positive genera, than against the gram-negative ones.

Favel et al. (1994) conducted antifungal screening of triterpenoid saponins of *Hedera helix* in vitro. Monodesmosidic hederagenin derivatives were shown to exhibit a broad spectrum of activity against yeast as well as dermatophyte species. α -hederin was the most active compound.

Elias et al. (1991) isolated from the leaves of *Hedera helix* four new triterpenoid saponins and characterized them by chemical and spectroscopic methods.

Danloy et al. (1994) analysed the effect of α -hederin, isolated from *Hedera helix* leaves on mouse B16 melanoma cells and non-cancer mouse 3T3 fibroblasts cultured *in vitro*. The results showed that, in a serum free medium α -hederin is cytotoxic and inhibit proliferation in both cell lines at a low concentration (< 5 μ g/ml) after only 8 hours treatment. α -hederin also induces vacuolization of the cytoplasm and membrane alterations, leading to cell death.

Quetin-Leclercq et al. (1992) reported that a crude extract of *Hedera helix* exert cytotoxic activity on Ehrlich tumor cells both *in vitro* and *in vivo*.

Kawagishi et al (1990) reported two cytotoxic phenols, hericenones A and B, isolated from mushroom *Herichium erinaceum*.

Rossi et al. (1991) reported an ellagic acid (a phenolic lactose) which is found in woody dicotyledonous

plants, being present in high concentration in nuts (e.g. Walnuts) and in fruits such as strawberries, raspberries, blackcurrants and grapes. This acid inhibits the carcinogenic properties of a variety of chemical compounds including Benz(a)pyrene-7,8-diol-9,10-epoxide, aflatoxin B₁, N-methyl-N-nitrosourea, 3-methyl-cholanthrene and 7,12-dimethylbenz(a)anthracene.

Takahashi et al. (1992) isolated a cytotoxic compound, Russuphelin A (2,6-dichloro-4-methoxyphenol) from the mushroom *Russula subnigricans*. He reported that Russuphelin A exhibited cytotoxic activity against 10 tumor cell lines including human derived carcinoma cells. He also reported (1993) three new compounds, Russuphelin B, C, and D isolated from the fruiting bodies of *Russula subnigricans* which exhibited cytotoxic activity *in vitro* against P388 leukemia cells.

Chen et al. (1994) studied the antitumor, antibacterial and wound healing properties of several species of *Croton* including *Croton lechleri* (which has

long been used in South American folk medicine, particularly in the treatment of wounds, cancer, inflammation and infection). He isolated several phenolic compounds from the sap of *Croton lechleri* which possess potent antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.

Marcias et al. (1994) reported slight antitumor activity of the phenylpropanoid compounds against 3 human carcinoma cell lines and P388, isolated from *Pimpinella villosa*.

Zeng et al. (1994) isolated 13 phenolic compounds from the stem bark of *Knema glomerata*, following bioactivity-directed fractionation against brine shrimp. All these compounds showed moderate but significant toxicities against 3 human tumor cell lines and inhibited the growth of crown gall tumors on discs of potato tubers.

Lin et al. (1994) isolated 11 phenolic compounds from the ethanolic extract of *Selaginella doederleinii*, a folk anticancer medicine. He reported that the cytotoxic activity of these compounds supported the traditional use of *Selaginella doederleinii* as an anticancer drug.

Blunden et al. (1994) reported the cytotoxic activity of higher molecular weight polyphenols, isolated from some marine algae (*Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus*, *Fucus spiralis*, *Halidrays siliquosea* and *Himanthalia elongata*).

Ramanathan et al. (1994) reported the antiproliferative effect of three plant polyphenols, 2-hydroxychalcone, quercetin and tannic acid.

Chen et al. (1994) isolated a phenolic compound, named isorawsonol from tropical green alga *Avrainvillea rawsonii*. The compound exhibited inhibitory activity against IMP dehydrogenase (IMPDH), a sign of potential anticancer activity.

Wu-Yc et al. (1995) reported the cytotoxic activity of some phenolic acid amides, N-trans-feruloyltyramine, N-p-coumaryltyramine and N-trans-caffeoyltyramine isolated from the stems of *Annona montana*.

1.3.1 DIABETES MELLITUS

It is a metabolic disorder characterized by hyperglycemia (above 180 mg/dl), glucosuria, thirst, hunger and in severe cases ketosis, related to inadequacy or abnormality of insulin production by pancreas. Many other factors are also involved, including both pituitary and adrenal hormones regulation.

Diabetes is one of the most prevalent diseases affecting mankind all over the world. The disease was even known to ancient peoples of Egypt, India and Greek. The Greek physicians examined the taste of the urine as an aid for the diagnosis. Avenenna (about 1000 A.D.) proved the association of gangrene with sugar in the urine (Monin 1987).

Diabetes mellitus is not only a serious health problem of the developed western countries, but it is also one of the most serious diseases in Pakistan. In Mexicans and Anglo Americans, the prevalence of the disease is 12% and 3% respectively (Rosental et al., 1984). In south Africa the prevalence in male is 7.6% and in female 13.5% (Omar et al., 1985). In India, the prevalence of this disease is 18% (Ahuja, 1978).

In Pakistan, diabetes mellitus is common in ages between 41-50 years in both the sexes, with slightly higher figures for females as compared to the males (Samad 1992).

There are two main types of "Diabetes".

i. Diabetes Insipidus.

It is rare and is due to defects in the pituitary gland. It is characterized by tasteless polyuria (5-10 Liters per day as compared to 1-1.5 Liters urine of normal person).

ii. Diabetes Mellitus.

It is the most common type of diabetes. It results from complete or relatively low production of insulin by the pancreas. It is characterized by hyperglycemia, glucosuria, thirst and in severe cases ketosis.

Diabetes mellitus is again of two types

- a. Type I or Insulin Dependent Diabetes Mellitus (IDDM).
- b. Type II or Non-Insulin Dependent Diabetes Mellitus (NIDDM).

Galloway (1988) reported that type I is often of early age onset (Javanile onset, generally before the age of 15). It is less common as compared to type II.

Type I diabetes mellitus results due to pancreatic β -cells distortion, with the resulting deficiency of

pancreatic insulin secretion, but normal sensitivity to insulin action.

The patients of this diabetes are usually thin and under weight. The two possible causes of β -cells destruction are the viruses, such as mumps virus and coxsackie by their toxic chemical agents or destructive cytotoxin and antibodies from sensitized immunocytes (Katzung, 1993). Insulin therapy is necessary for the control of type I diabetes mellitus.

Type II, (NIDDM) :

According to Galloway (1988), type II diabetes mellitus is more common type and usually occurs in middle aged persons, where most of the sufferers have obesity. Weight loss is uncommon. Patients have usually normal plasma insulin level, but a deficiency of insulin receptors on the muscle and fat cells results in decreased sensitivity to insulin. There is a strong genetic predisposition of the development of type II diabetes mellitus. This type of diabetes occurs due to over eating

of rich caloric diets (fats and carbohydrates) and lack of physical exercise. These patients always have some impairment in insulin secretion. However an increase in resistance to insulin action is more marked. This type of diabetes is controlled with diet management and oral hypoglycemic drugs.

Diabetes mellitus is a serious incurable, chronic disease, that carries a high risk for a considerably short life span, renal failure, disability, complications including blindness, imputation of organs, renal failure, sexual impotence and early cardiovascular complications.

1.3.2 TREATMENT OF DIABETES MELLITUS

Diet is an important factor for treatment of diabetes mellitus. Rogers and Spector (1976) described that Carbohydrates restriction and weight loss restore glucose tolerance to normal level to many of the non insulin

depended diabetic patients. Similar conclusion has been made by Ebodi (1993).

Oral Hypoglycemics

Herfindal (1988) reported that the oral hypoglycemics drugs are used in NIDDM patients, who fail to achieve adequate control despite adhesion to prescribed diet and exercise.

There are two main groups of oral hypoglycemics drugs.

i. Sulphonylureas.

These drugs act by stimulating the production and release of indigenous insulin (Herfindal 1988) e.g., Tolbutamide , Acetohexamide and Glipizide.

ii. Biguanides

These drugs act by reducing appetite and glucose uptake by the intestine and promoting glucose utilization mainly in muscles e.g. Metformin and Phenformin .

INSULIN

It is a specific antidiabetic principal and obtained from bovine source or through genetic engineering from the two mutant strains of *E. coli* (Bacteria). Insulin is commercially available in several different forms.

1.3.3 ANTIDIABETIC ACTIVITY IN PLANTS

Many plants, plant extracts and natural products, isolated from various plants have been screened for their hypoglycemic activity. In some instances the desired effects were found, whereas some time the significant hypoglycemic effect was not observed. A brief review of recently reported hypoglycemic plants is given below.

Khan *et al.* (1980) reported that the leaf of *Coccinia indica* improved the glucose tolerance considerably.

Akhter *et al.* (1981) reported significant hypoglycemic effect of *Momordica charantia* in normal and alloxan diabetic rabbits.

Karunanayake et al. (1984) reported that the aqueous decoction of *Salacia reticulata*, *Aegle marmelos*, and *Momordica charantia* possess significant hypoglycemic effects.

Lamela et al. (1985) have observed significant hypoglycemic activity of ether extracts of *Lythrum salicaria* stem and flowers in rats.

Jimenez et al. (1986) demonstrated the hypoglycemic activity of *Salvia lavandulfolia* in alloxan diabetic rabbits.

Akhtar et al. (1986) observed significant hypoglycemic effect of dried roots of *Onosama echinoides* in both normal as well as diabetic rabbits.

Tomoda (1987) reported the hypoglycemic activities of mucilages extracted from plants of family Malvaceae and some *Plantago* species.

Ivorra et al. (1988) demonstrated hypoglycemic effect of tormentic acid, a natural product isolated from *Poterium ancistroides* in both normal and diabetic rates.

Wadood et al. (1988) observed significant hypoglycemic effect of alcoholic extract of leaf of *Eriobotrya japonica*.

Day et al. (1990) reported hypoglycemic effect of orally administered extracts of fruits of cultivated *Momordica charantia* in normal and streptozotocin diabetic mice.

Shanmugasunaram et al. (1990) reported the antidiabetic activity of *Gymnema sylvestra*.

DeTommasi et al. (1991) reported hypoglycemic effect of leaf extracts of *Eriobotrya japonica* in both genetically diabetic and normal rats.

Gonzalez et al. (1991) studied the hypoglycemic activity of *Olea europaea* leaf extract and proposed that hypoglycemic activity of this plant is by their mechanisms of (i) potentiation of glucose induce release of insulin, and (ii) increased peripheral uptake of glucose.

Srivastava et al. (1993) found highly hypoglycemic effect of *Momordica charantia* in diabetic patients.

1.3.4 DIABETES AND TRACE ELEMENTS

Trace elements play vital role in health and disease. Some naturally occurring elements are essential for life, other are non-essential and some of them are known to be toxic at all levels in their occurrence in biological systems (Mertz, 1981).

The essential elements are vital for the proper functioning and growth of various organisms (Nielsen, 1982).

Schoeder (1976) described that less than 0.02% (or 11 gm) of the reference man is made up of trace elements. At least 24 trace elements are found in human body in amounts large enough to be detected. Eleven of the twenty four are of vital importance for his health. He also explained that most of these trace elements act as catalyst (Co-enzyme to Co-factor). These elements are combined with proteins (enzymes), making metallo-enzymes for example Magnesium activates 67 enzymes. Various clinical and pathological disorders arises as a consequence of trace element deficiencies and excesses.

Donsbach (1982) reported that man can exist on a deficiency or even absence of certain vitamins but can not exist on an extreme deficiency or an absence of certain minerals in such away that particular element create problem while conducting deficiency experimentation.

Nowadays there is considerable evidence to show that the deficiency of some of the trace elements may accompany diabetes.

Chromium (Cr)

It has been reported that Chromium is an essential part of dietary factor. Schroder (1968), Mertz (1969) and Masironi (1973) demonstrated that a severe Chromium depletion led to fasting hyperglycemia, glycosuria and impaired growth rates in rats. Also serum cholesterol levels of these animals were elevated compared with those Chromium supplemented controls and an increased incidence of aortic plaques had been reported. Chromium supplementation of rats fed diets of refined sugars, which are associated with elevated serum cholesterol levels have also been reported to decrease significantly these cholesterol levels. Mertz (1969) investigated that even mild nutritional Chromium deficiency in rats results in impairment of Glucose tolerance. He added that small quantities of Chromium are required for the optimal effect of insulin in every insulin dependent system. Mertz et al. (1989) also reported that the beneficial effects of Chromium are related to a useable form of Chromium and not the total Chromium. This useable form of Chromium may be

present in foods, as such, or can be biosynthesized within the body by most people.

Janjua(1991) analysed the hair samples of diabetic patients and found that, in most of the cases Chromium and Zinc concentrations were much lower as compared to those of normal persons

It is known that an organo-chromium complex, Glucose Tolerance Factor (GTF), containing glycine, glutamic acid, cysteine and niacin, is synthesized in tissues from absorbed Chromium. This complex potentiates the action of insulin through facilitating the attachment of insulin to cell membranes, that ultimately enhance the uptake of glucose by the cells (Uusitupa et al., 1983 and Robinson et al., 1985). The deficiency of this complex, either through absolute dietary lack of Chromium or reduced ability to synthesize that specific complex, results in glucose intolerance and reduced efficiency of insulin. symptomatic Chromium deficiency has been clearly demonstrated in man during prolong intravenous feeding

when purified nutrients (Amino acids and Carbohydrates) which do not contain sufficient Chromium to meet its requirement.

In two studies, supplementation of intravenous fluid with 250 μg Chromium per day , or intravenous supplementation with 150 μg Chromium per day reversed severe glucose intolerance, improved nitrogen balance and weight gains (Ottaway *et al.*, 1986).

Zinc (Zn)

Huber and Gershoff (1973), Kirchgessner *et al.* (1976) and Wolman (1979) demonstrated that abnormalities in glucose utilization that occur with severe Zinc depletion are related to impaired release of insulin from pancreas and to a decline in peripheral tissue sensitivity to insulin.

Lee *et al.* (1989) reported that oral zinc is able to increase the intestinal absorption of glucose and vice

versa. He reported that there is an interaction between Zinc and insulin metabolism.

Manganese (Mn)

Donsbach (1982) described that rats fed with a Manganese deficient diet for two months, showed a diabetic or prediabetic glucose curve, and it stayed up there. When the rats were put back on Manganese adequate diet, their glucose tolerance curve returned to normal. That showed that the body was not utilizing glucose in the absence of adequate Manganese.

Amjad et al. (1993) reported that the blood glucose lowering activity of chromium citrate, zinc citrate and manganese gluconate has been investigated in normal and alloxanised rabbits by treating them for 20-25 days respectively. The data obtained suggested that the hypoglycemic effect of these minerals was less marked in normal rabbits, than the hypoglycemic effect in the diabetic rabbits. The salts can be ranked in the

decreasing order of hypoglycemic activity as chromium citrate, manganese gluconate and zinc citrate.

In view of the important role of Chromium, Zinc and Manganese in the glucose metabolism, it is decided to analyze *Hedera helix* leaf for these trace elements.

1.4 DESCRIPTION OF *HEDERA HELIX*

Bot. name: *Hedera helix* L

Syn: *Hedera nepalensis* K.Koch

Family: Araliaceae

Eng. name: Ivy, Barren Ivy, creeping Ivy.

Local names: Ur-Bumbal (Hazara), Lablab (Hindi), Banda (Punjabi), Karmora (Kashmiri), Phalool (Pushto).

Habitat

Plant is found over a greater part of Europe, Northern and central Asia (Grieve, 1974). In Pakistan it grows wild in Kurrum, Swat, Hazara, Murree hills and Kashmir from 5-9 thousand feet (Stewart, 1972). It is a common climber on *Pinus wallichiana* trees of Murree-Hazara hills (plate-1). *Hedera helix* also grows as creeper over the ground in woods.

1.4.1 DESCRIPTION

Hedera Helix is an evergreen climber with dark-green, glossy and angular leaves. It climbs up by means of



Plate-1: *Hedera helix*, growing in their natural habitat
(Murree-Hazara hills).

adventitious roots, which shoot out from every part of the stem and are furnished with small disks at the end. These disks then adopt themselves to the roughness of the bark or wall against which the plant grows and to which it clings firmly. These clinging roots on meeting with the soil or deep crevices become true roots obtaining nourishment for the plant. The stem of the climber plant is 1 - 2cm in diameter (plate-2), but when left to grow in protected places, like grave yards, the plant forms a very thick (5 - 20cm) trunk (plate-3 & -4).

After attaining the maximum height (20-30 ft), the plant grows out in a bushy form and the leaves instead of being five lobed and angular, as they are below, become ovate with entire margins. There is all the variation in the leaf shape from angular palmatipartite incision to complete entire margins as the plant attains height.



Plate-2: *Hedera helix* climber plant, leaves removed from upper part of the plant to show the thickness of the stem.



Plate-3: An old *Hedera helix* plant (right), with a very thick trunk, growing on olive tree in a graveyard of Abbottabad.



Plate-4: *Hedera helix* plant (about 30-35 years old), with thick trunk, growing on pine tree in the Department of Botany, University of Peshawar.

The creeper plant has very thin stem, being 2 - 3mm in diameter. The creeper plant has only the true roots, arising from the lower side of the stem and it bears long petiolate leaves, which are uniform in structure, being triangular, with 3 - 5cm lobes (plate-5). Creeper plants do not flower, but on coming in contact with trunks of supporting trees, they climb up by means of adventitious roots and become climber plants (plate-6). The plant produces flowers on the terminal branches. The flowering branches being bushy and projecting a foot or two from the climbing stems with flowers at the end of every shoot. The flowering period is from late October to late December. Inflorescence is a globular umbel. The flowers are yellowish green. Calyx entire with 5-sepals, corolla with 5-petals, valvate. Stamens 5, carpels 5, syncarpous, pentalocular. Fruit is a berry, becomes ripe in the next spring. It is 5-7 mm long and 5-10 mm broad with 2-5 seeds (fig-1).



Plate-5: Creeper *Hedera helix* plant.



Plate-6: Creeper *Hedera helix* plant becoming climber on coming in contact with a supporting tree.

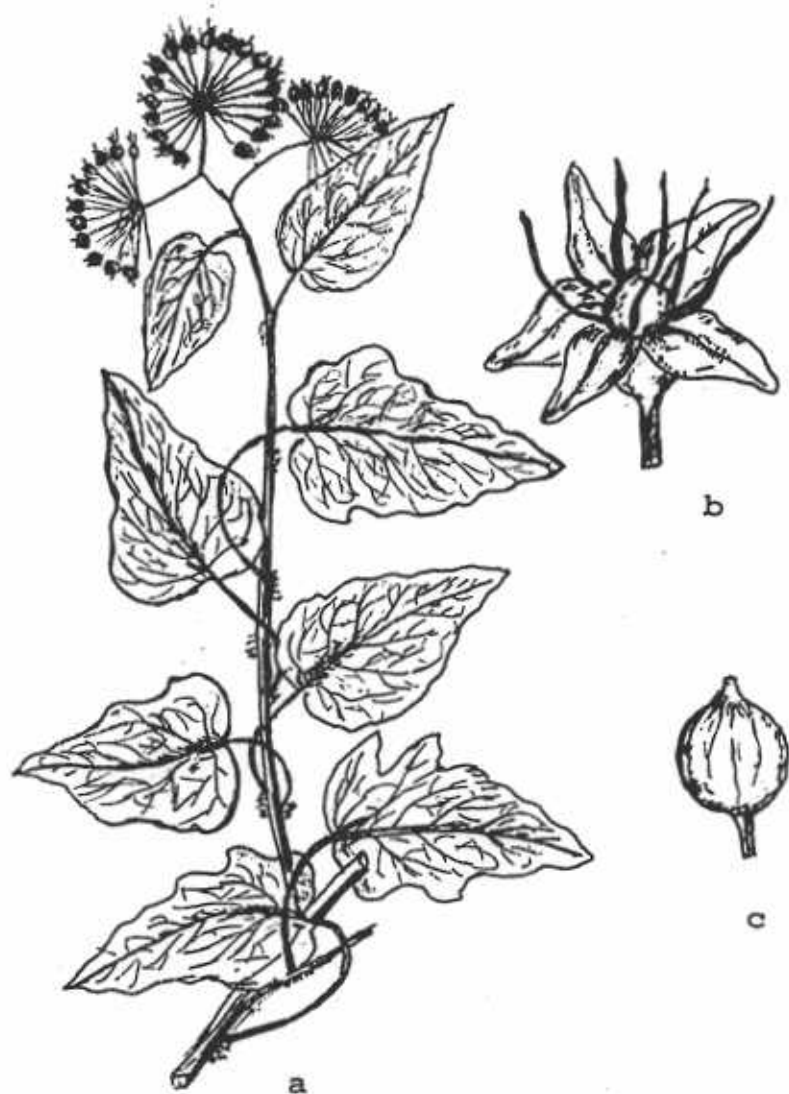


Fig. 1: *Hedera helix*, a. Habit x 1/2; b. Flower x 6; c. Fruit x 2.

CHAPTER-II

MATERIALS AND METHODS

CHAPTER-II

MATERIALS AND METHODS

2.1 PHARMACOGNOSTIC STUDY OF *HEDERA HELIX* L.

2.1.1 PLANT MATERIAL

Leaves of *Hedera helix* L. were collected from Bara gali, District Abbottabad, in the month of August, 1995. The leaves were dried in shade.

2.1.2 CHEMICALS

Stains and reagent used for staining, chloral hydrate solution, Jeffrey's solution (equal parts of 10% aqueous nitric acid and 10% aqueous chromic acid), Hydrochloric acid, acetic anhydride, ferric sulphate, pet. ether.

2.1.3 INSTRUMENTS

Microscope (4mm, 16mm and 45mm objectives and x10 Eye-piece), Blade, stage and Eye piece micrometers, camera lucida, electric balance, kiln.

2.1.4 ORGANOLEPTIC EVALUATION OF THE DRUG

Organoleptic evaluation, including shape, size, colour, venation, odour and taste of the *Hedera helix* leaves will be carried out.

2.1.5 HISTOLOGICAL STUDIES OF *Hedera helix* LEAF

2.1.5.1 Transverse section:

Fresh leaves of *Hedera helix* were used for section cuttings. A small stripe (2m x 15mm) from the midrib region of the leaf was taken, clamped within potato block and with the help of sharp blade transverse sections were cut. A thin unruptured section was selected and stained. This stained transverse section was traced with the help of camera lucida and labeled.

For epidermal study, stomatal index, vein islet number and palisade ratio, small strips of the leaf (about 5mm x 15mm) were cut with sharp scalepal, washed with water and than boiled and cooled repeatedly to free them from air and then soaked in Jeffrey's solution for 6 days (Johanson 1940) for maceration and disintegration of

tissues. Even after 8 days, the tissues were not clear and not easily disintegrated. These strips were then boiled in chloral hydrate solution on a water bath (Trease, 1983). This cleared the strips and when examined under microscope, gave excellent sight of the epidermis. With slight adjustment of the microscope, the underlying palisade cells were also visible for examination.

2.1.5.2 Epidermis:

Leaf strips treated with Jeffrey's and chloralhydrate solutions were examined under microscope using 45mm objective and X10 eye piece. Both the upper and lower epidermal cells were measured with the help of micrometers and traced with the help of camera lucida.

2.1.5.3 Palisade ratio:

Palisade ratio is the average number of palisade cells, beneath each upper epidermal cell (Trease, 1983).

Method:

The cleared strips of leaves were examined under microscope, using 4mm objective and X10 eye piece (Trease 1983). A camera lucida is so arranged, that the epidermal cells may be traced. The palisade cells of the mesophyll bears a definite ratio to the epidermal cells. This ratio is a diagnostic character of species belonging to the same genus (Wallis, 1985).

First four groups, each of four epidermal cells are traced and their outline linked to make them more conspicuous. The palisade cells lying beneath each group are then focused with fine adjustment of the microscope, and traced. The palisade cells in each group were counted. Only those cells were included in the count, which were more than half covered by the epidermal cells.

The figure obtained divided by four gave the palisade ratio of *Hedera helix* leaf.

2.1.5.4 Stomatal index:

Stomatal index is the percentage, that the stomata form of the total number of epidermal cells and stomata. It is expressed by the formula

$$I = \frac{S}{E+S} \times 100$$

I = Stomatal index

S = Number of stomata in unit area

E = Number of epidermal cells in the same unit area.

Method:

The cleared leaf strips about 5mm were mounted using 45mm objective and X10 eye piece . Both the epidermal cells and stomata were counted. The two guard cells and osteol, being considered as one unit (Trease 1983). A cell being counted, if at least half of its area has within the field of view. Stomatal indices of four cleared strips, belonging to apex, margin, base and centre of the leaf were determined and the mean stomatal index was calculated.

2.1.5.5 Vein islet number:

Vein islets are areas of the green tissue of leaf, formed by the ultimate divisions of the conducting strands of vascular bundles, which either completely or partially surround areas of the chlorenchyma.

Method:

Cleared strips, about 4mm from different positions (apex, margin, base and centre) of five matured leaves were examined under compound microscope having a 16mm objective and X10 eye piece. The area of the vein islets was determined by counting the number of vein islets within the field of vision (app. 2mm^2), divided by the area of the field (2mm^2) this gave the average number of vein islets per unit area (Youngken, 1948). Vein islet number of four different positions (apex, centre, margin and base) of five mature leaves was determined and from those the average vein islet number of *Hedera helix* leaf was determined.

2.1.5.6. Powdered drug study:

Dried leaves of *Hedera helix* were powdered to a very fine state in an electric grinder.

Organoleptic study of the powder drug was carried out using the sense organs. For microscopic study, a small amount of the powder was heated with chloralhydrate (200%) solution for few minutes and a very little amount of this chloralhydrate treated powder was transferred to microscopic slide and a drop of glycerin was added to avoid drying. A coverslip was put on the specimen and the slide was observed under microscope. Different cells and tissues in the powder were seen and sketched with the help of camera lucida.

2.2 CHEMICAL TESTS

2.2.1. ASH ANALYSIS

Ash analysis, specially of powdered drugs is very important to detect the addition of adulterants in the form of earthy or sandy material or for the detection of

exhausted materials added to the genuine drug. Three different ash analysis were carried out.

Determination of Total Ash:

Three grams powdered leaves of *Hedera helix* were taken in a crucible and incinerated at 450°C for about one hour in a kiln to obtain carbon free ash. The crucible was taken out and after cooling, weight of the ash was determined. From this value the percentage of the ash with reference to the air dried powder drug was calculated. Three replicates for this test were taken and the average value was calculated.

Determination of Acid Insoluble Ash:

The total ash obtained as above was boiled for five minutes with 25 ml of dilute hydrochloric acid. It was then filtered using ashless filter paper (Whatman No 41). The filter paper was washed with hot water and ignited to a constant weight. Percentage of acid insoluble ash was

calculated with reference to the dried powdered leaf (3 gm).

Determination of Water Soluble Ash:

Ash was obtained as in (I) above. The ash was boiled in 25 ml distilled water and after cooling filtered through ashless filter paper (Whatman No 41). The filter paper was washed with hot water and ignited to a constant weight at a temperature not exceeding 450°C to obtain water insoluble ash. It is subtracted from the total ash weight to obtain the weight of water soluble ash. Percentage of the water soluble ash was calculated with reference to the dried powdered leaf (3 gm).

2.2.2 Detection of Calcium oxalate:

A small quantity of the leaf powder was taken on a slide and few drops of a saturated solution of cupric acetate were added and allowed to undergo chemical reaction for ten minutes. Then few drops of a solution (prepared by dissolving 2.5 gm ferric sulphate in 50 ml of

20% acetic acid) were applied to the slide and observed under the microscope. Yellow colour crystal if appeared, would indicate the presence of calcium oxalate crystals in the leaf of *Hedera helix* (Gur, 1956).

2.2.3 Screening Test for the Presence of Triterpenoidal Saponins (Liebermann Burchard Test)

Alcoholic leaf extract was taken in a petridish and evaporated to dryness. The dried residue was washed many times with pet.ether on a hot water bath in order to remove fatty and colouring materials. 10 ml chloroform was added to the residue and poured into a test tube. 100 mg of anhydrous sodium thiosulfate was added to the test tube and filtered. 3 ml of the filtrate was taken in a small test tube and three drops of the acetic anhydride and one drop of concentrated sulfuric acid were added.

The appearance of Red violet colour would indicate the presence of triterpenoidal saponins in the leaf powder of *Hedera helix* (Brain and Turner, 1975).

2.3 ANTIDIABETIC STUDY OF *HEDERA HELIX*:

PLANT MATERIAL USED

Leaves of *Hedera helix* were collected from Baragali in the month of September. The leaves were dried in shade. After garbling and removing petioles the dried leaves were powdered in grinder. The powdered leaves were then stored in a well closed amber glass bottle at room temperature.

Preparation Of Aqueous Extract:

200 gm of the powdered leaves of *Hedera helix* were macerated in 1000 ml distilled water for 7 days at room temperature and the extract was then filtered. The aqueous filtrate was concentrated through rotary evaporator under vacuum to a semi solid mass (40.00 gm).

Preparation of Methanolic Extract:

Methanolic extract was prepared, using the same procedure, as adopted for the aqueous extract. After removing methanol under vacuum, 48.42 gm of semisolid mass was obtained.

The aqueous and methanolic extracts were used orally for the determination of their antidiabetic activity in the test animals (rabbits).

2.3.1 CHEMICALS/REAGENTS:

Alloxan monohydrate	(BDH. Lab, England)
Methanol	(Merck, Germany)
Acetohexamide (Demirol)	(Lilly/Ali Gohar Pharmaceutical (Pvt.) Ltd. Karachi, Pakistan)
Metformin (Glucophage)	(Boots Pharmaceuticals Ltd. Karachi, Pakistan.
Gum tragacanth	(Pharmacy Deptt., University of Peshawar)
Easy Test Strips:	Rreagent strip for glucose testing in capillary whole blood (Cat.No.00560,Boehringer Mannheim Corp. USA) .

2.3.2 INSTRUMENTATION:

Glucometer (Accu-Check Easy Blood Glucose Monitoring System. Cat.No.00560, Boehringer Mannheim Corp. USA,).

Lancet (Cat.No.00585, Boehringer Mannheim Corp. USA)

Syringes 20 ml and Polythene Tube.

EXPERIMENTAL ANIMALS:

Healthy rabbits (*Oryctolagus cuniculus*) of local strain weighing 1000-1500 gms were purchased from Secondary Board and Bacha Khan Chowk markets of Peshawar. These animals were kept under observation for one week before experimentation under usual management condition and fed with normal diet consisting of green grass during this period. The rabbits were then randomly divided into following three groups.

Group-I: 6 rabbits, control group

Group-II: 18 rabbits, normal, non diabetic group.
This group is further sub divided in to three groups.

Group-IIa: 6 rabbits, tested for aqueous extract of *Hedera helix*.

- Group-IIb: 6 rabbits, tested for methanolic extract of *Hedera helix*.
- Group-IIc: 6 rabbits, tested for acetohexamide (Dimerol)^R an oral hypoglycemic drug.
- Group-III: 18 rabbits, alloxan-induced diabetic group. This group is also further sub divided in to three groups.
- Group-IIIIa: 6 rabbits, tested for aqueous extract of *Hedera helix*.
- Group-IIIIb: 6 rabbits, tested for methanolic extract of *Hedera helix*.
- Group-IIIIc: 6 rabbits, tested for metformin (Glucophage)^R an oral hypoglycemic drug.

2.3.3 INDUCTION OF ALLOXAN-DIABETES:

Alloxan causes β -cell necrosis which is preceded by an initial inhibition of insulin release. Within few hours, hypoglycemia occurs due to excessive discharge of insulin from dying pancreatic cells.

Mechanism of Action of Alloxan:

The exact mechanism is not yet fully known. Various possible mechanisms, proposed, are as under.

1. Alloxan acts by destroying β -cells of the islets of Langerhans, possibly by breaking DNA strands. Alloxan appears to generate free radicals and causes inactivation of essential sulphhydryl enzyme of the cells, without damaging α -cells (acinous cells) of the pancreas (Katzung, 1993).
2. Alloxan binds instantly to islet cell membrane and causes rapid, *in vitro* and *in vivo* inhibition of the insulin secretory mechanism (Gordsky, 1982).

3. The removal of Zinc from insulin in the form of chelates by alloxan results in the diabetogenic effect (Burger, 1960).
4. Alloxan inactivate Ca^{++} and calmoduline dependent protein kinase, which reduces insulin secretion (Katzung, 1993).

INDUCTION OF ALLOXAN-DIABETES IN RABBITS OF GROUP-III:

Rabbits of group-III were made diabetic artificially by injecting alloxan monohydrate 150 mg/Kg body weight in the marginal ear vein (Butt, 1962). Before injecting alloxan, blood glucose level of these rabbits were determined with the help of Glucometer, to exclude rabbits with abnormal blood glucose levels.

Eight days after injecting alloxan monohydrate, the blood glucose level of the surviving rabbits were determined and those having blood glucose level of 250-400 mg/dl were considered as diabetic and were employed for further study.

PROCEDURE

The rabbits of Group-I (Control) were given orally 20 ml of 2% Gum Tragacanth solution with the help of polythene tube connected with 20 ml syringe. Blood glucose level at 0, 2, 4, 8, 12 and 24 hours were recorded (Appendix-1).

The rabbits of Group-IIa and IIb were given aqueous extract and methanolic extract respectively at dose equivalent to 4 gm of powdered leaf per Kg body weight in 20 ml 2% gum tragacanth solution and blood glucose levels at different time intervals were recorded (Appendix-2 & -3).

Rabbits of Group-IIc received oral hypoglycemic drug, Acetohexamide, in 20 ml of 2% gum tragacanth solution at a dose of 500 mg/Kg body weight. The blood glucose level was recorded at different time intervals (Appendix-4).

The rabbits of Group-IIIa and IIIb (alloxan-diabetic) were given aqueous extract and methanolic extract

respectively at dose equivalent to 4 gm of powdered leaf per Kg body weight in 20 ml 2% gum tragacanth solution and blood glucose levels at different time intervals were recorded (Appendix-5 & -6). The rabbits of Group-IIIC were given metformin, equivalent to 500mg per Kg. body weight in 20ml 2% gum tragacanth solution. The blood glucose level at different time intervals were recorded (Appendix-7).

2.3.4 PROCEDURE OF BLOOD GLUCOSE LEVEL DETERMINATION

A small marginal area of the ear of the rabbits was shaved off to expose the skin, this area was cleaned with alcohol wipe and when the alcohol was dried, the rabbit was laid down on the margin of the table in such a position that the ear hung downward in order to increase blood flow to the ear. In this position a prick was made on the exposed skin with the help of lanced device, and with little pressure of the fingers a drop of blood oozed out.

Glucometer was turned on. From the air tight pack a test strip was taken out. The blood drop was gently

touched to the center of the target area (Target area of the strips contain test reagents) of the reagent strip. When the blood fully absorbed into the strip and the target area was no longer shiny, the strip was inserted into the glucometer, with the target area facing the dark red spot on the glucometer.

The readings appeared within 15-60 seconds as mg/dl on the screen and were recorded.

TEST PRINCIPLE:

The determination of glucose in capillary blood with Easy Test Strip is based on the glucose oxidase/ferricyanide reaction, coupled with the formation of potassium ferricyanide, when whole blood is placed on the strip, β -D-glucose is oxidized to α -D-gluconolactone and potassium ferricyanide is reduced to potassium ferrocyanide with glucose oxidase acting as catalyst. The potassium ferrocyanide then combines with ferric ion, present in the test pad to produce the indicator prussian

blue. The intensity of the color formed is proportional to the glucose concentration in the blood specimen.

2.4 ELEMENTAL ANALYSIS OF THE PLANT MATERIAL

2.4.2 REAGENTS

All the solutions were prepared from analytical grade chemicals using ultrapure double-distilled water.

- a. Hydrochloric acid (36%) E.Merck
- b. Nitric acid (65%) E.Merck
- c. Perchloric acid (70%) E.Merck

2.4.2 INSTRUMENTATION

The trace elements were determined by flame photometry, using SP 191 PYE UNICAM A.A.S., in the Geochemistry Laboratory of the Centre of Excellence in Geology, University of Peshawar.

PREPARATION OF PLANT SAMPLES:

Surface contamination of plant material was removed by brushing and brief rinsing in distilled water. The

entire surface was gently rubbed in 0.3 N hydrochloric acid and rinsed. The sample was rapidly dried in a forced-draft oven at 70°C.

The sample was ground by means of a grinder. Mixed well and stored in tightly stoppered containers.

Two methods, Dry Ashing and Wet Digestion, are commonly used for the determination of trace elements in plant materials (Issac et al, 1975). In the present work, both the methods were employed and the average of the two results were calculated.

2.4.3 DRY ASHING PROCEDURE:

One gram of dried, grounded plant material was weighed and placed in a porcelain crucible. It was then placed in a cool muffle furnace and ashed at 500°C overnight. The ash was cooled and dissolved in 5 ml of 20% hydrochloric acid. This solution was then filtered through an acid-washed filter paper into 50 ml volumetric flask,

the filter paper was also washed twice. The solution was diluted to volume with double distilled water.

Aliquots of this solution were used for the determination of Chromium.

2.4.4 WET DIGESTION PROCEDURE:

One gram of ground plant sample was taken and placed in a small beaker. Then 10 ml of concentrated nitric acid was added and allowed to stand over night. It was then heated on hot plate untill the production of red fumes of NO_2 had ceased. The beaker was cooled and a small amount (2-4 ml) of perchloric acid was added in it. It was again heated and allowed to evaporate to a small volume. This was then transferred to a 50 ml flask and dilute to volume with double distilled water and filtered through a dry filter paper without washing.

This solution was retained and used for analysis.

DETERMINATION OF ELEMENTS (Cr, Zn & Mn) :

A. PREPARATION OF STOCK/STANDARD SOLUTIONS (1000 ppm)

1. CHROMIUM (Cr)

Accurately weighed 3.725 gm of K_2CrO_4 was dissolved in deionized water and diluted to one liter with double distilled water.

2. ZINC (Zn)

One gram zinc metal was dissolved in about 20 ml of (1:1) HCl and was diluted to one liter with double distilled water.

3. MANGANESE (Mn)

One gram of Mn metal was dissolved in concentrated HCl and was made to the volume of one liter with double distilled water.

B. PREPARATION OF STOCK/STANDARD SOLUTIONS (100 ppm)

10 ml of 1000 ppm standard stock solution of each

element was taken in 100 ml volumetric flask and was made to the volume with double distilled water.

C. WORKING STANDARD SOLUTIONS (0.5, 1, 2, 4 and 8 ppm)

Working standard solutions: 0.5, 1, 2, 4 and 8 ppm of working standards were prepared by taking 0.5, 1, 2, 4 and 8 ml of 100 ppm stock solution in a series of 100 ml volumetric flasks and volume was made to the mark with double distilled water.

2.4.5 DETERMINATION OF CHROMIUM:

INSTRUMENT CONDITIONS:

Mode	Setting
Wave length	357.9 nm
Slit width	0.4 nm
Acetylene flow	4.5 L/min
Air flow	4.8 L/min
Cathode lamp current	9.0 mA

PROCEDURE:

The atomic absorption spectrophotometer was set according to the above mentioned conditions. Chromium cathode lamp was turned on and allowed to warm up for 10 minutes. After warming up of cathode lamp the air acetylene flame was ignited. The instrument was calibrated and standardized with working standards of 0.5, 1, 2, 4 and 8 ppm. All the working standards were then run as unknown to verify the standardization. Stock solutions of certified standards were run through the instrument and the results were compared with the certified values. After making sure that the results of the certified standards were within the confidence limit, the sample stock solutions were then aspirated into the flame and the Chromium concentration of the sample was noted.

The Chromium contents in ppm were calculated.

2.4.6 DETERMINATION OF Zn

INSTRUMENT CONDITIONS:

Mode	Absorption
Wavelength	213.9 nm

Slit width	0.4 nm
Acetylene flow	1.2 L/min
Air flow	4.3 L/min
Cathode lamp current	10.0 mA

PROCEDURE:

The atomic absorption spectrophotometer was set according to the above mentioned conditions. Zn cathode lamp was turned on and allowed to warm up for 10 minutes. After warming up of cathode lamp the air acetylene flame was ignited. The instrument was calibrated and standardized with working standards of 2, 4 and 8 ppm. All the working standards were then run as unknown to verify the standardization. Stock solution of certified standards were run through the instrument and the results were compared with the certified values. After making sure that the results of the certified standards were within the confidence limit, the sample stock solutions were then aspirated into the flame and the Zn concentration of the sample was noted.

The Zn contents in ppm were then calculated.

2.4.7 DETERMINATION OF Mn:

INSTRUMENT CONDITIONS:

Mode	Absorption
Wavelength	279.5 nm
Slit width	0.4 nm
Acetylene flow	1.2 L/min
Air flow	4.2 L/min
Cathode lamp current	12.0 mA

PROCEDURE:

The atomic absorption spectrophotometer was set according to the above mentioned conditions. Mn cathode lamp was turned on and allowed to warm up for 10 minutes. After warming up of cathodes lamp the air acetylene flame was ignited. The instrument was calibrated and standardized with working standards of 2, 4 and 8 ppm. All the working standards were then run as unknown to verify the standardization. Stock solution of certified standards were run through the instrument and the results were compared with the certified values. After making sure that the results of the certified standards were within

the confidence limit, the sample stock solutions were then aspirated into the flame and the Mn concentration of each sample was noted.

The Mn contents in ppm were calculated.

2.5 PHYTOCHEMICAL AND CYTOTOXIC STUDIES OF *HEDERA HELIX*.

2.5.1 MATERIAL

Leaves of *Hedera helix* were collected from Baragali, Abbottabad, Maindam-Swat Marghuzar-Swat and from Botany Department, University of Peshawar in the month of September. The leaves were washed to remove adhering dirt and were then dried in shade. After garbling and removing petioles the dried leaves were powdered using hand driven mincing machine. The powdered leaves were then stored in a well closed amber glass bottle at room temperature.

Silicagel Kieselgel 60 for Column Chromatography.

Silicagel Kieselgel 60HF254 for TLC.

2.5.2 CHEMICALS

Ethanol, methanol, chloroform, n-hexane, n-butanol and ether. (All analytical grades.)

2.5.3 PREPARATION OF METHANOLIC EXTRACT:

Five hundred grams of the powdered leaves of *Hedera helix* were macerated in 1250 ml methanol for 7 days at room temperature and the extract was then filtered. The residue left was again soaked in methanol and extracted after 7 days. The two methanolic extracts were combined and after vacuum evaporation, 87.5 gm of a semisolid extract (E1) was obtained (Harborne, 1973).

Fourty grams of E1 was repeatedly washed with petroleum spirit to remove coloring and fatty material. A brittle mustard colored, 21.8 gm extract (E2) was obtained.

2.5.4 FRACTIONATION OF METHANOLIC EXTRACT (E1)

Fifteen grams of E1 was dissolved in 150 ml of 1:1 chloroform and distilled water in a separating funnel. The

mixture was shaken out at different intervals for 24 hours and then allowed to separate. The lower chloroform fraction (E1F1) was separated and evaporated in rotary vacuum (2.88 gm). The interphase of the two liquids (E1F2), 0.4 gm, was also collected. The upper aqueous layer (E1F3) was also evaporated in rotary vacuum to a semisolid mass (1.30 gm).

Fraction E1F1 (2.88 gm) was sub fractionated with aqueous-methanol (9:1) and n-hexane. Two fractions, a lower aqueous-methanolic (E1F4) (1.1 gm), and upper n-hexane fraction (E1F5) (0.78 gm), were obtained.

To obtain further fractions of E1, 15 gms of E1 were again taken in separating funnel along with 150 ml 1:1 mixture of chloroform and aqueous-methanol (9:1). After shaking for 24 hours at regular intervals, it was allowed to stand. The lower chloroform fraction was separated, evaporated under vacuum and a semisolid (2.84 gm) chloroform fraction thus obtained was added to E1F1 and the blackish pitch, 0.96 gm, at the interphase was also

collected and added with similar pitch E1F2 of the first separation. To the remaining aqueous-methanol part, 75 ml ethylacetate was added, shaken at regular intervals for 24 hours and after standing, the ethylacetate fraction (E1F6), was separated, evaporated in rotary vacuum to semisolid mass (2.1 gm).

To the remaining aqueous-methanol fraction, 75 ml of n-butanol was added, shaken for regular intervals in the separating funnel and after standing, the n-butanol fraction (E1F7) was separated and evaporated in rotary vacuum to semisolid mass (2.42 gm) and the remaining aqueous-methanolic fraction after vacuum evaporation (2.14 gm) was added with E1F4.

The following fractions were obtained from the crude methanolic extract (E1).

- | | |
|------------------------------|------|
| 1. Chloroform Fraction | E1F1 |
| 2. Interphase Fraction | E1F2 |
| 3. Aqueous Fraction | E1F3 |
| 4. Aqueous-methanol Fraction | E1F4 |

5.	n-Hexane Fraction	E1F5
6.	Ethylacetate Fraction	E1F6
7.	n-Butanol Fraction	E1F7

2.5.5 FRACTIONATION OF METHANOLIC EXTRACT (E2) :

The same procedure was adapted for the fractionation of E2 and the fractions thus obtained were named as E2F1 - E2F7 respectively.

2.5.6 EXTRACTION OF SAPONIN FROM THE LEAVES OF *HEDERA*

HELIX

Initially the following three different methods of saponin extractions were tested to find out the most feasible method that would be adopted for further detail research work.

Method No 1:

Sixty grams of the powder leaves of *Hedera helix* were soaked in methanol (450 ml) for seven days at room temperature and then filtered. The filtrate was evaporated at reduced pressure in the rotary evaporator to a thick

residue. This residue was repeatedly washed with petroleum spirit to remove the chlorophyll and fatty materials. The process was continued till there were no traces of colouring matter in the petroleum spirit. The residue obtained was hard brittle and mustard coloured, which crumpled to powder form by the use of slight pressure.

This powder was redissolved in 100 ml methanol. To this methanolic solution ether solvent was added drop wise. A white precipitate of Saponin was formed. The addition of ether was continued, untill no further precipitate was formed. The precipitate was recovered by decantation (Harbore, 1973).

The yield of saponin by this method is 3.93%.

Method No 2:

Sixty grams of powder leaves of *Hedera helix* were first extracted with chloroform (300 ml) in a soxhlet apparatus for 24 hors. The residue left was next extracted with ethanol (300 ml) for 24 hors in soxhlet apparatus. The ethanolic extract was evaporated in vaccum to a

rasidue which was next dissolved in methanol (100 ml). To this methanolic solution ether solvent was added drop wise to precipitate the saponins as in the method No 1 (Bikram Singh et al., 1987).

The yield of saponins by this method is 4.66%.

Method No 3:

Sixty grams of the powder leaves of *Hedera helix* were soaked in 300 ml methanol:water (60:40) for 7 days. The extract was filtered and evaporated in rotary evaporator to get a residue. To the residue, methanol (100 ml) was added. To this methanolic solution ether solvent was added to precipitate the saponins, as applied in method No 1 and 2, the precipitate obtained was next partitioned between water and n-butanol (100:100) in a separating funnel. The saponin were retained in upper organic layer. The organic layer was separated and evaporated in a rotary evaporator (Elias et al., 1991). The yield of saponin by this method is 1.46%.

As the percent yield of saponin by method No 2 was better than the other two methods, therefor, it was adopted for the extraction of saponin for further work.

Saponin were extracted by method No 2 from the leaves of *Hedera helix* collected from different places of N-W.F.P. The saponin of different places were tested for cytotoxcity using Brinshrimp bioassay.

The part of leaf extract, named as "RESIDUE", left after isolation of saponin was retained, methanol and ether were evaporated from it and was also tested for cytotoxicity.

Only the "RESIDUE" was found to be cytotoxic. It was further fractionated through column chromatography using chloroform, mehtanol:chloroform (1:1) and methanol as mobile phases. The three fractions were evaporated and tested for cytotoxicity.

The chloroform fraction showed significant cytotoxicity. In order to isolate the cytotoxic principle this chloroform fraction was further fractionated using preparative TLC. Various combinations of different solvents as mobile phase were tried in order to develop a suitable mobile phase.

The n-Hexan:chloroform: methanol system (1:1:2.5) was adopted as it separated the sample into six clear bands, F1 - F6. The Rf values of these bands were determined. The bands were then scrapped and collected separately.

This process of preparative TLC was repeated many times to run about 400 mg of the sample. The scratched fractions were extracted from silica gel with the help of n-hexane, chloroform and methanol respectively. The solvents from these fractions were evaporated and the weight of dried fractions (F1-F6) were recorded.

These fractions were then tested for cytotoxicity using the same Brinshrimp bioassay.

Out of these six fractions only the fraction F4 showed some significant cytotoxicity.

In order to know the chemical nature of fraction F4 chemical test, UV and IR spectroscopies were performed.

2.5.7 BRINESHRIMP BIOASSAY

Brineshrimp Hatching

Sea salt solution was prepared by dissolving 38 gm sea salt in 1000 ml double distilled water and final solution thus prepared was filtered. The solution was taken in a small plastic tub which was divided by a partition, having small holes in it. The eggs were sprinkled on one side of the partition, which was then covered with a black carbon paper. The other half of the tub was illuminated with an electric lamp. After 48 hours the shrimp hatched and matured as nauplii. They actively swam and migrated through the holes in the partition wall to the illuminated side of the tub.

Bioassay Procedure:

Twenty milligrams of each of the extracts/fractions was taken in small vial and dissolved in 2 ml of methanol to serve as stock solution.

From the stock solution, 500 μ l, 50 μ l and 5 μ l (corresponding to 1000, 100, 10 μ g/ml respectively) were transferred to vials with three replicate of each concentration. The vials were placed uncovered for 24 hours for complete evaporation of the solvent, methanol. After complete evaporation of the solvent 2 ml sea salt solution was added to each of the vial. 10 brineshrimp were transferred to each vial (30 brineshrimps per dilution) with the help of long-tipped dropper and the volume was adjusted to 5 ml with the sea salt solution.

After 24 hours, survived brineshrimps were counted for all the concentrations of the extracts/fractions and isolated Saponins (Tables -4 to -11, -13 to -15, & -17).

2.5.8 LC₅₀ CALCULATION

LC₅₀ were determined from the 24 hours death counts of brineshrimps, using the Probit analysis method or Finney computer programme (Finney, 1981).

2.6 ANTIBACTERIAL ACTIVITY:

The isolated Saponin of *Hedera helix* were tested against some common pathogenic and nonpathogenic bacterial strains, applying Disc diffusion technique (Kirby-Bauer, 1960), at Sibro Pharma (Pvt.) Nowshera.

PROCEDURE:

Commercially available filter paper discs were sterilized in the autoclave and then impregnated with specific quantity (10.0 mg/ml) of the test sample. These discs were then applied on to the surface of the agar plates over which a culture of the microorganism had been streaked. After 18 hours of incubation at 37°C, the size of a clear zone of inhibition around the disc was determined.

This procedure was adopted for all the test organism and the results are given in Table-18.

CHAPTER-III

RESULTS

CHAPTER-III

RESULTS

3.1 PHARMACOGNOSTIC STUDY OF *HEDERA HELIX*

3.1.1 MORPHOLOGIC DESCRIPTION OF LEAVES

Leaves alternate, simple petiolate, petiole 3 to 14 cm long, the leaves glabrous, dark green on the upper side and green on the lower side, with reticulate venation. The leaves of the climber plants are highly heterophyllous, 4-16 cm long and 3-8 cm broad. The lower leaves are smaller, obscurely 3-5 lobed (Figs 2a-2d). Those on the upper part of the plant becomes less and less incised, and ovate to ovate-lanceolate with no incision at all (Figs 2e-2g).

The leaves of creeper plants are dark green, uniform in shape, being triangular with 3 or 5 lobes, 8-12 cm long and 5-8 cm broad. The petioles of the creeper plants are longer (10-22cm) than the climber leaves (3-14cm). The venation is reticulate, but the lateral veins along the midrib join to form 2-4 conspicuous heart-shape structures

(fig 2h). Fresh leaves have no odour, while dried leaves have senna-like odour. Leaves are slightly bitter in taste. Fresh leaves have a tough texture, while dried leaves are brittle.

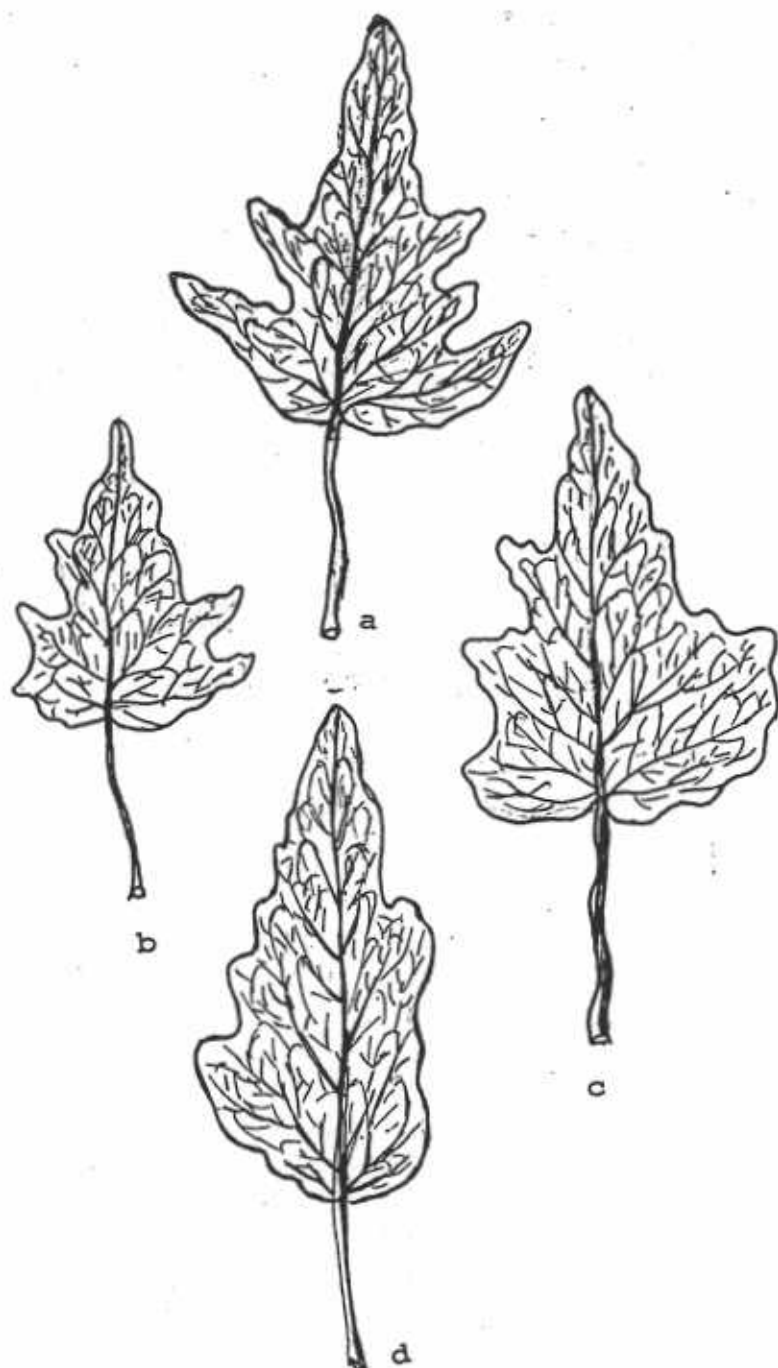


Fig. 2: *Hedera helix* leaves. a. $\times 1\frac{1}{2}$; b. $\times 1\frac{1}{2}$ - $\times 2\frac{1}{2}$; c. $\times 1$ - $\times 2$; d. $\times 1\frac{1}{2}$ - $\times 2\frac{1}{2}$.

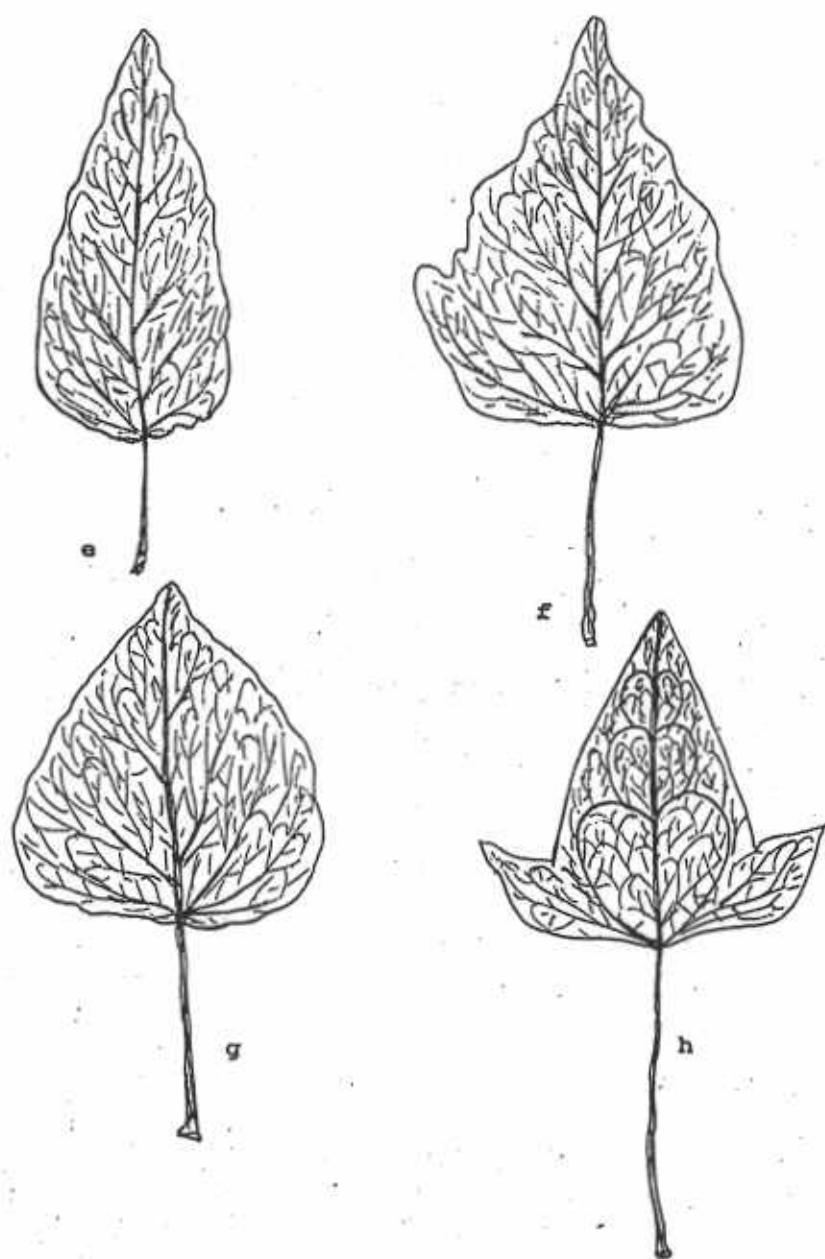


Fig. 2: *Hedera helix* leaves. e. $\times 1\frac{1}{2}$ - $\times 2$; f. $\times 1\frac{1}{2}$ - $\times 2\frac{1}{2}$; g. $\times 1\frac{1}{2}$ - $\times 2\frac{1}{2}$; creeper leaf h. $\times 1$ - $\times 1\frac{1}{2}$.

3.1.2. Transverse section of the leaf

Transverse section of the *Hedera helix* leaf (Fig. 3) shows bifacial arrangement. Both the upper and lower epidermis is covered with cuticle. There are no trichomes on both the surfaces. Stomata are present only on the lower epidermis. Below the upper epidermis is a multilayered (3-4 celled) palisade parenchyma. In the midrib region palisade parenchyma is replaced by collenchyma on both the sides.

Spongy parenchyma cells are large, green, and many of them contain rosette aggregates of calcium oxalate crystals. Some of these crystals are very large and occupy the whole space within the cell.

The midrib is composed of vascular bundle, which is almost completely surrounded by pericyclic fibers. Phloem lies inner to pericycle and consists of very thin walled sieve tubes. Inner to the phloem is xylem, consisting of radiating xylem tracheids.

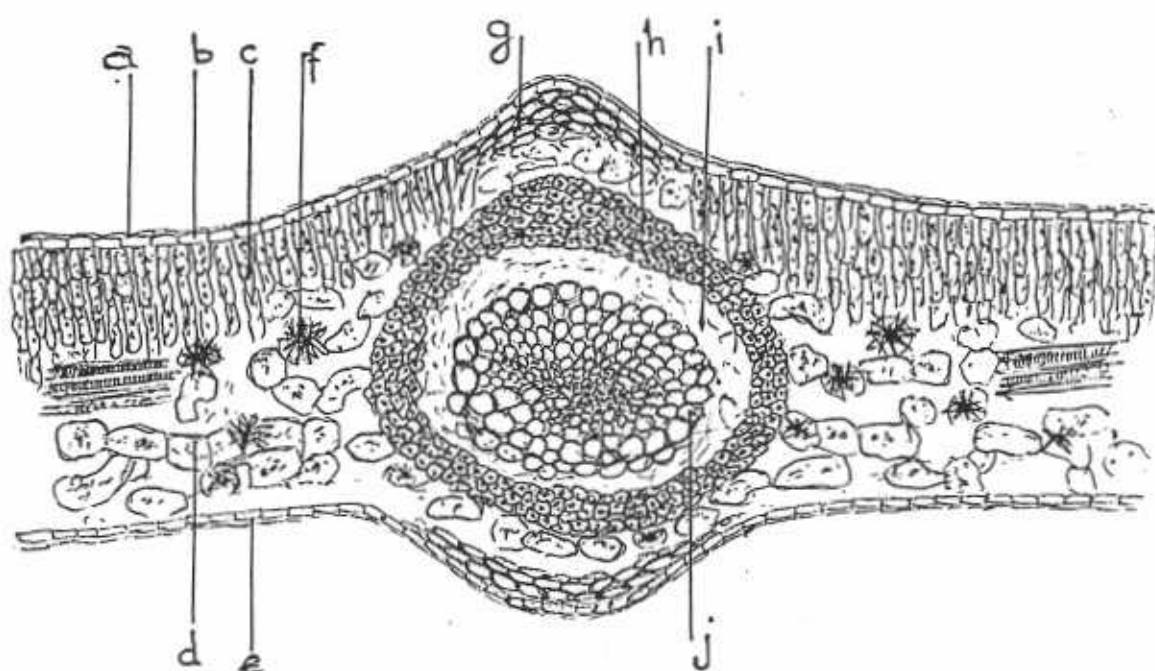
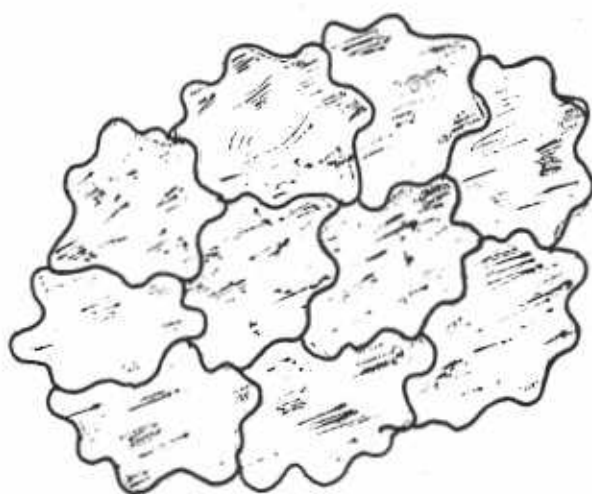


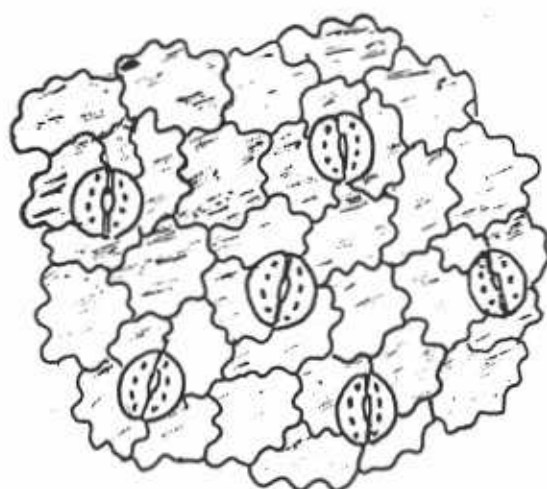
Fig. 3: Transverse Section of *Hedera helix* Leaf.
 a. cuticle, b. upper epidermis, c. palisade
 parenchyma, d. spongy parenchyma, e. lower
 epidermis, f. rosette aggregate of calcium
 oxalate, g. collenchyma, h. pericycle,
 i. phloem, j. xylem.

3.1.3 Epidermis

Jaffrey's solution could not clear the leaf strips, these were then heated in chloral hydrate solution (200%), which cleared these strips. The microscopic observations were made, using 45m.m. objective and X10 eye piece (Youngken, 1948). The upper epidermal cells (Fig. 4a) are undulated and larger (24-36 μ m) than the lower epidermal cells which are 21.5 to 28.8 μ m (Fig. 4b). The lower epidermal cells are also undulated. Stomata are present only on the lower epidermis. Stomata are large (20-25 μ m) and orbicular. Each stoma is surrounded by 3-4 subsidiary cells, which are smaller than rest of the lower epidermal cells.



a



b

Fig. 4: *Hedera helix* leaf, a. Upper epidermis; b. Lower epidermis.

3.1.4. Palisade Ratio:

There are about 5-6 palisade cells under each group of four epidermal cells. Four groups of epidermal cells (each with four cells), along with their underlying palisade cells were counted as per procedure (Trease, 1983). Six palisade cells each were attached to the first and fourth groups, while five palisade cells each were attached to the second and third groups of the epidermal cells. The average palisade ratio as calculated from the reading comes out to be 1.4.

3.1.5 Stomatal index:

Stomata were present only on the lower epidermis. The number of stomata/mm² field of view, counted from the cleared strips belonging to base, margin, centre and apex of the leaf were 13, 12, 15 and 14 respectively while the number of epidermal cells at these positions were 107, 113, 105 and 116 respectively, all these figures are the average of three readings. The stomatal index at these positions were calculated using the following formula;

S

$$I = \frac{S}{E + S} \times 100$$

Where I = Stomatal Index

S = Number of stomata/mm²

E = Number of epidermal cells/mm²

The mean stomatal index of *Hedera helix* leaves (Tab - 1) ranges from 9.6 - 12.5 with an average of 10.9.

3.1.6 Vein islet number:

The vein islet number at different positions (base, margin, centre and apex) of five different mature leaves of *Hedera helix* were determined (Tab-2). The average vein islet number at these positions were 12.2, 14.4, 10.4 and 12.6 respectively. As a whole the average vein islet number of *Hedera helix* leaf is 12.4 with a range of 10.4 to 14.4.

Table-1: Data of the Stomatal Index at different positions of *Hedera helix* leaf.

S.No	Position	Number of stomata/mm ² (S)	Number of epidermal cells/mm ² (E)	Stomatal Index (I)
1	Base	13	107	10.9
2	Margin	12	113	9.6
3	Centre	15	105	12.5
4	Apex	14	116	10.7

Table-2: Average number of vein islet numbers at different positions of *Hedera helix* leaf.

S.No	Position	Leaf					Average
		1	2	3	4	5	
1	Base	12	16	9	11	13	12.2
2	Margin	10	13	15	16	18	14.4
3	Centre	10	9	12	10	11	10.4
4	Apex	10	12	15	10	12	12.6

3.1.7 Powdered drug study of *Hedera helix* leaf

The organoleptic evaluation of the powdered leaf of *Hedera helix* was carried out as listed below.

Colour	-	Light Green
Odour	-	Senna- like
Taste	-	Slightly bitter

Microscopic evaluation of the powdered leaves of *Hedera helix* was carried out using X10 eye piece and 16 mm objective. The chloral hydrate treated powder of *Hedera helix* leaf showed the various cells/tissue, which are sketched (Fig 5). These include the following.

Fragments of both the upper and lower epidermis. The upper epidermal cells are 24-36 μm and the lower epidermal cells 21.5 - 28.8 μm with orbicular stomata.

Spongy parenchyma cells are 12-18 μm and irregular in shape. Many of these cells contain rosette aggregate crystals of calcium oxalate .

Fragments of upper epidermis, with attached palisade cells. Fragments of tracheids 5-8 μm in diameter and with compact spiral thickening. Fragments of pericycle fibers 3-4 μm in breadth and fragments of collenchymatous cells.

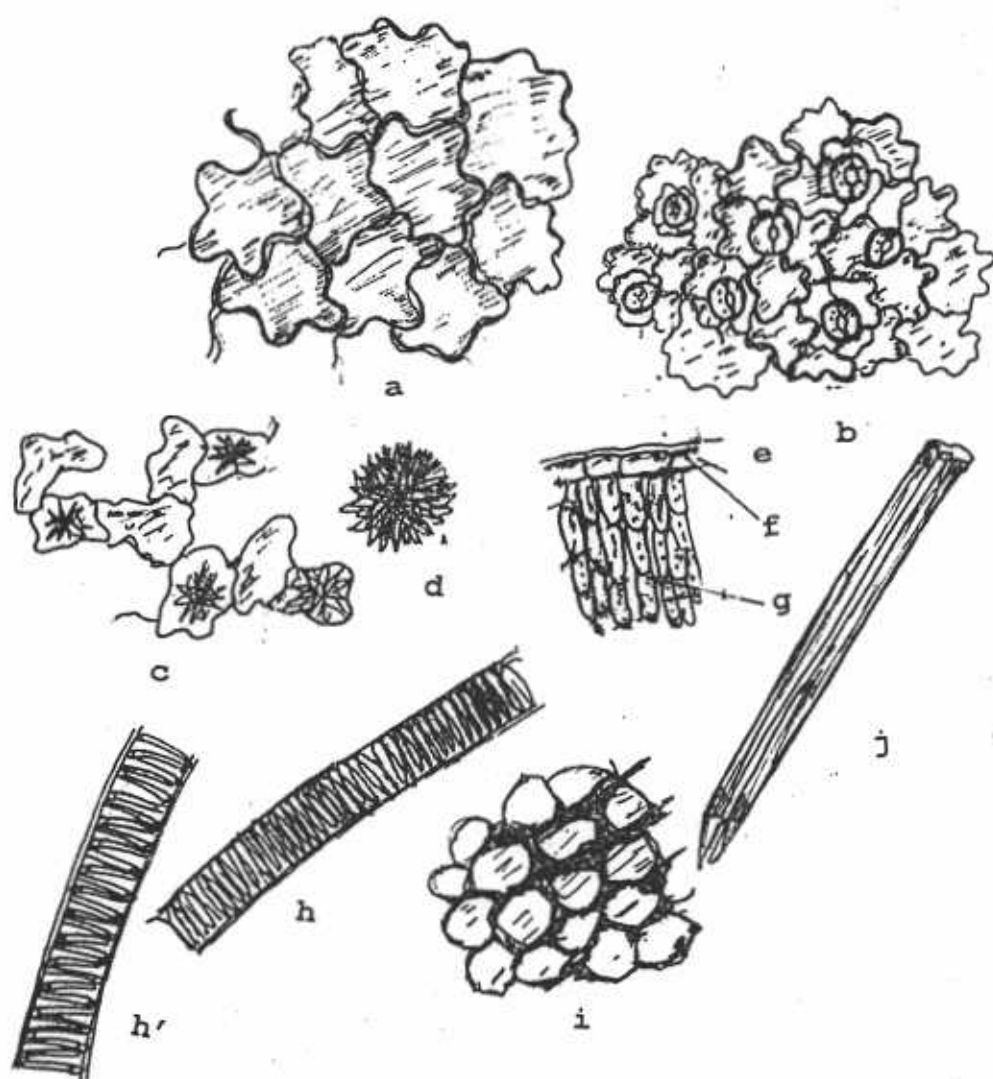


Fig. 5: Powder drug of *Hedera helix* leaf. **a** & **f**. Upper epidermis; **b**. Lower epidermis; **c**. Spongy parenchyma; **d**. Rosette aggregates of Calcium oxalate crystals; **e**. Cuticle; **g**. Palisade parenchyma; **h** & **h'**. Trachea; **i**. Collenchyma; **j**. Fiber.

3.2 CHEMICAL TESTS

3.2.1. Ash Analysis:

Total ash of the genuine (un-exhausted) leaf powder (3.0gm) is 0.24gm (8.33%), its water soluble is 0.62gm (2.07%) and its acid insoluble ash is 0.019gm (0.63%).

The total ash of the exhausted leaf powder (3.0gm) is 0.18gm (5.86%) and its water soluble ash is 0.03gm (0.96%).

3.2.2. Detection of Calcium oxalate

Calcium oxalate was determined in the *Hedera helix* leaf as per procedure (Gur, 1956). When cupric acetate solution was added to the powder, the crystals of calcium oxalate got dissolved within 8-10 minutes. When ferric sulphate solution was added to this, the dissolved crystals of calcium oxalate changed into crystals of ferrous sulphate and turned into yellow colour, confirming the presence of calcium oxalate in *Hedera helix* leaf.

3.2.3 Screening Test for Triterpenoid Saponin (Liebermann-Burchard Test) :

Liebermann-Burchard test (Brain and Turner, 1975) was performed for the detection of triterpenoidal saponins. Red violet colour appeared, showing the presence of triterpenoidal saponins in *Hedera helix* leaves.

3.3 ANTIDIABETIC (HYPOGLYCEMIC) STUDIES OF *HEDERA HELIX*

Data obtained after the oral administration of aqueous and methanolic extracts of *Hedera helix* equivalent to 4 gm of powdered leaves per Kg body weight and acetohexamide, 500 mg/Kg body weight (Table-3, appendixes 1-4) clearly show that both extracts have hypoglycemic effects at a significant level ($P < 0.05$).

It is evident from table-3 that aqueous extract started hypoglycemic effect at 4 hour time interval after the oral administration whereas the methanolic extract started its effect at 8 hour time interval. At 8 hour time interval in both the cases the decrease in blood glucose

level became statistically significant ($P < 0.005$). At 12 hour time interval, the hypoglycemic effect of the aqueous extract remained significant ($P < 0.005$) while that of methanolic extract became less significant ($P < 0.05$). At 24 hour time interval, the blood glucose level in all the cases again rose to its maximum.

As for as the hypoglycemic drug, acetohexamide is concerned its effect (highly significant) started at 2 hour time interval and remained highly significant upto 8 hour time interval.

The overall comparison of the hypoglycemic effect of the aqueous and methanolic extracts of the powdered leaves of *Hedera helix*, acetohexamide and control is given in fig. 6.

The administration of aqueous and methanolic extracts of *Hedera helix* leaves along with an oral hypoglycemic drug, Metformin to alloxan-induced diabetic rabbits showed that both the aqueous and methanolic extracts exhibited

hypoglycemic effect (table-4, appendixes 5-7). Hypoglycemic effect of both the extracts started at 8 hour time interval but as compared to the methanolic extract, ($P < 0.05$), the aqueous extract showed highly significant ($P < 0.005$) effect at 8 hour time interval and sustained upto 12 hour time interval, where as the effect of methanolic extract is less significant at these time intervals. The effect of metformin started at 2 hour time interval ($P < 0.005$) and sustained up to 12 hour time interval. the over all comparison of the two extract and metformin is given in fig.7

The flamephotometry analysis of the powdered leaf of *Hedera helix* (tab-5) for the hypoglycemic trace elements (Cr, Mn, and Zn) showed that it contain Chromium 15.50 ppm, Manganese 53.25 ppm and Zinc 49.75 ppm.

TABLE-3 DATA OF BLOOD GLUCOSE LEVEL (BGL) OF NORMAL RABBITS GROUP-I (CONTROL) RECEIVED 2% GUM TRAGACANTH SOLUTION AND GROUP-IIa, GROUP-IIb & GROUP-IIc, RECEIVED AQUEOUS AND METHANOLIC EXTRACTS EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWERED LEAVES OF *Hedera helix* AND ACETOHEXAMIDE, 500mg/Kg BODY WEIGHT IN 2% GUM TRAGACANTH SOLUTION, RESPECTIVELY.

TIME INT. (hrs)	GROUP-I (CONTROL)	GROUP-IIa Aqueous Extract	GROUP-IIb Methanolic Extract	GROUP-IIc Acetohexa- mide
0	94.17±0.60	92.00±0.57	91.83±0.87	92.16±0.83
2	94.00±0.58	90.00±0.57	91.00±0.51	79.66±1.76**
4	94.33±0.81	88.33±0.92*	90.83±0.80	76.50±1.82**
8	93.50±0.67	82.66±0.84**	87.83±0.79**	81.00±0.85**
12	91.67±0.50	83.33±1.12**	90.66±0.33*	87.16±0.75**
24	91.33±0.56	92.33±0.84	93.00±0.68	92.00±0.57

* Significant decrease as compared to zero hour level (P<0.05)

** Highly significant decrease as compared to zero hour level (P<0.005).

Number of animals for each observation = 6

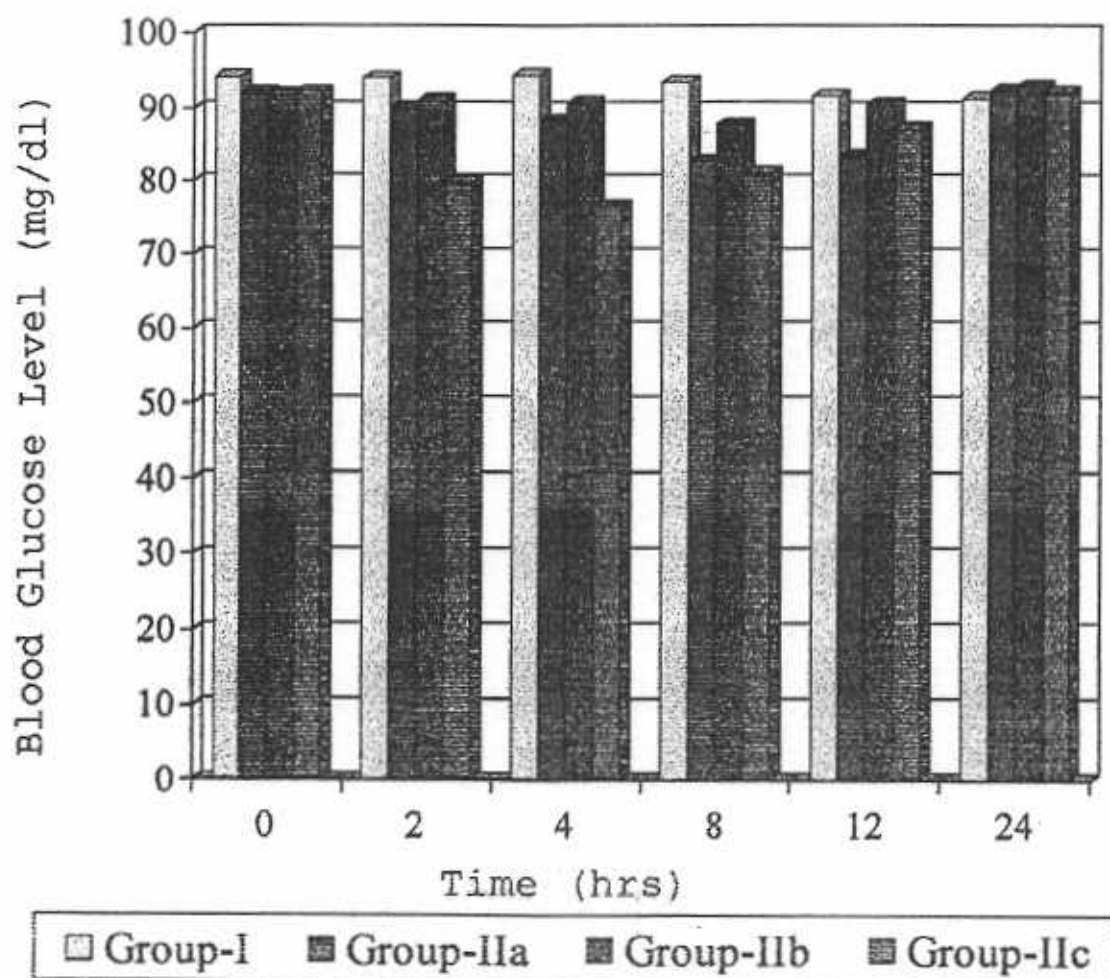


Fig-6 Blood Glucose level Vs Time interval of Normal Rabbits.

TABLE-4: DATA OF BLOOD GLUCOSE LEVEL (BGL) OF ALLOXAN-INDUCED DIABETIC RABBITS (GROUP-IIIa, GROUP-IIIb & GROUP IIIc) RECEIVED AQUEOUS AND METHANOLIC EXTRACTS EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWERED LEAVES OF *Hedera helix* AND METFORMIN 500 mg/Kg BODY WEIGHT IN 2% GUM TRAGACANTH SOLUTION, RESPECTIVELY.

TIME INT. (hrs)	GROUP-IIIa Aqueous Extract	GROUP-IIIb Methanolic Extract	GROUP-IIIc Metformin
0	257.17±3.14	260.50±2.86	261.50±2.40
2	255.00±2.61	260.00±2.59	238.00±2.83**
4	251.66±2.26	259.00±2.92	225.67±1.57**
8	245.66±2.15**	251.50±3.25*	217.83±3.63**
12	242.80±2.09**	254.16±2.24*	245.17±3.50**
24	262.50±5.33	262.00±2.45	262.17±2.80

* Significant decrease as compared to zero hour level (P<0.05)

** Highly significant decrease as compared to zero hour level (P<0.005).

Number of animals for each observation = 6

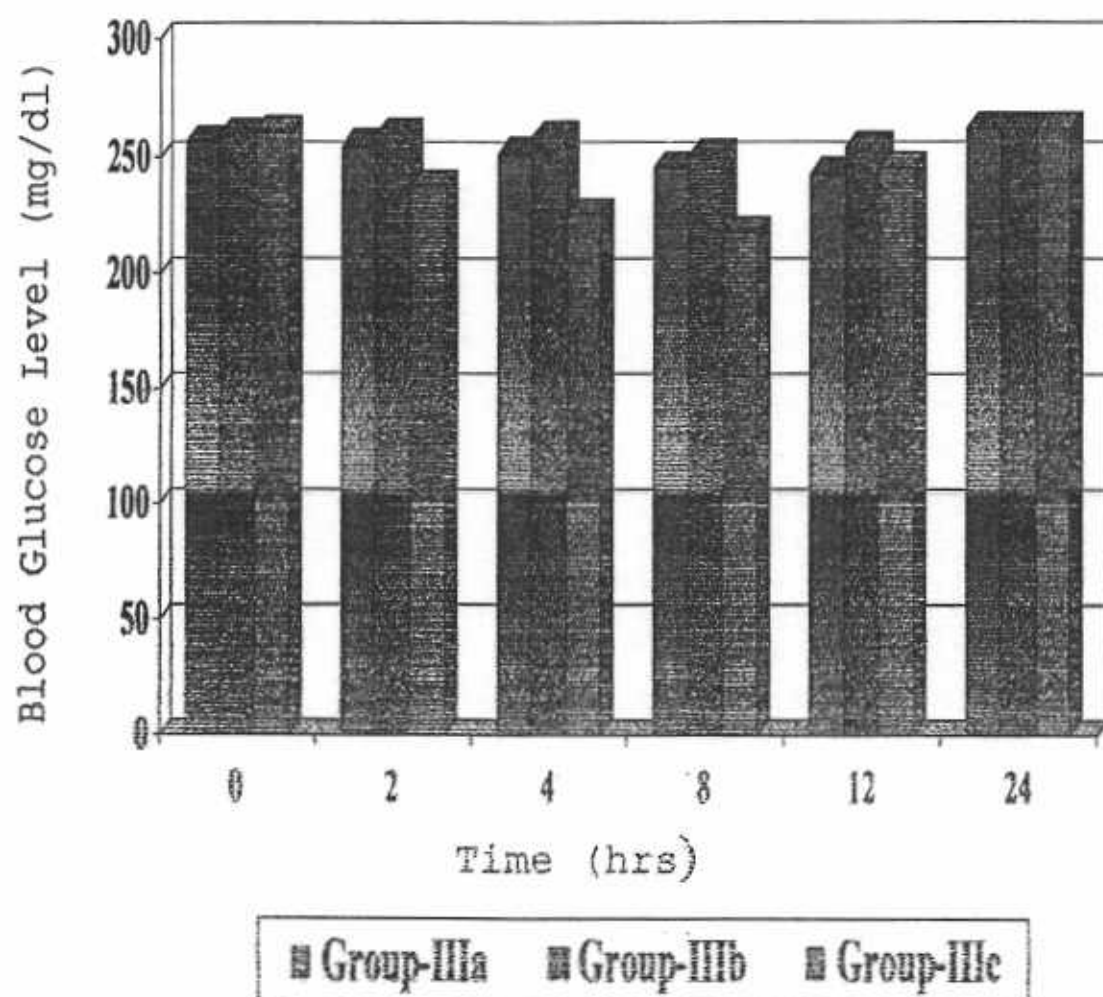


Fig-7 Blood Glucose level Vs Time interval of Alloxan induced Diabetic Rabbits.

TABLE-5: DATA OF THE TRACE ELEMENTS PRESENT IN THE
POWDERED LEAF OF *Hedera helix*.

Trace Elements	Concentration (ppm)
Chromium (Cr+++)	15.50
Zinc (Zn++)	49.75
Manganese (Mn++)	53.25

3.4 PHYTOCHEMICAL AND CYTOTOXIC STUDIES OF *HEDERA HELIX*

The methanolic extract (E1 & E2) of dried powdered leaves of *Hedera helix* and their different fractions (E1F1-E1F7 & E2F1-E2F7 respectively) were screened for their cytotoxic activity using Brineshrimp bioassay. The result of these bioassays are given in tables 6-13.

From the given data, it is clearly evident that only the two methanolic extracts (E1 & E2) and the chloroform fraction of E2, i.e. E2F1 exhibited some what significant cytotoxicity (Table-6 & 7), where as rest of the fractions of both the extracts were found to be inactive (Table-8 to 13) as their $LC_{50} > 1000 \mu g$.

The cytotoxic activity of chloroform fraction E2F1 (LC_{50} 700.24 μg) and methanolic extract E2 (LC_{50} 802.73 $\mu g/ml$) is more significant as compared to the methanolic extract E1 (LC_{50} 942.71 $\mu g/ml$).

The saponin isolated from the leaves of *Hedera helix* (both Climbers and creepers) collected from

different places of N-W.F.P is given in Table-14. From the table it is evident that there is no effect of climate and height on the saponinic content of *Hedera helix*. The saponinic contents are from 3.41 - 4.97% with an average of 4.32%.

The Saponin isolated from plants of various places of N-W.F.P. were tested for their cytotoxicity, the result of brineshrimp bioassay (Table-15) showed that the Saponin of *Hedera helix* are not cytotoxic ($LC_{50} > 1000 \mu g$) and that there is no difference in the composition of Saponins regarding their cytotoxic potential.

The bioassay result (Table-16) of the fraction left after separation of saponin ,i.e., the "RESIDUE", is significant ($LC_{50} 700.54 \mu g$), indicating that this part of the extract of dried leaves of *Hedera helix* contains the cytotoxic principles.

In order to isolate the cytotoxic principle from this "RESIDUE" it was further fractionated through column chromatography. The fractions were eluted using chloroform, chloroform:methanol(1:1)and methanol solvents.

Bioassay data (Table-17) of these three fractions obtained through column chromatography further revealed that the cytotoxic principle is present in the chloroform fraction (LC_{50} 423.59 μ g), where as the other two fractions(2&3) are not cytotoxic, as their LC_{50} are greater than 1000 μ g.

TABLE-6: DATA OF BIOASSAY OF CRUDE METHANOLIC EXTRACTS
(E1 & E2).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁</u>				
1	1000.0	30	16	942.71
2	100.0	30	8	
3	10.0	30	6	
<u>E₂</u>				
1	1000.0	30	15	802.73
2	100.0	30	10	
3	10.0	30	3	

TABLE-7: DATA OF BIOASSAY OF CHLOROFORM FRACTIONS
(E1F1 & E2F1).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₁</u>				
1	1000.0	30	6	>1000
2	100.0	30	3	
3	10.0	30	2	
<u>E₂F₁</u>				
1	1000.0	30	18	700.24
2	100.0	30	4	
3	10.0	30	2	

TABLE-8: DATA OF BIOASSAY OF INTERPHASE OF AQUEOUS-CHLOROFORM FRACTIONS (E1F2 & E2F2).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₂</u>				
1	1000.0	30	5	>1000
2	100.0	30	3	
3	10.0	30	2	
<u>E₂F₂</u>				
1	1000.0	30	6	>1000
2	100.0	30	3	
3	10.0	30	2	

TABLE-9: DATA OF BIOASSAY OF AQUEOUS FRACTIONS (E1F3 & E2F3).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₃</u>				
1	1000.0	30	5	>1000
2	100.0	30	4	
3	10.0	30	2	
<u>E₂F₃</u>				
1	1000.0	30	7	>1000
2	100.0	30	4	
3	10.0	30	3	

TABLE-10: DATA OF BIOASSAY OF AQUEOUS-METHANOLIC FRACTIONS (E1F4 & E2F4).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₄</u>				
1	1000.0	30	7	>1000
2	100.0	30	6	
3	10.0	30	2	
<u>E₂F₄</u>				
1	1000.0	30	9	>1000
2	100.0	30	7	
3	10.0	30	4	

TABLE-11: DATA OF BIOASSAY OF n-HEXANE FRACTIONS (E1F5 & E2F5).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₅</u>				
1	1000.0	30	6	>1000
2	100.0	30	2	
3	10.0	30	2	
<u>E₂F₅</u>				
1	1000.0	30	8	>1000
2	100.0	30	3	
3	10.0	30	2	

TABLE-12: DATA OF BIOASSAY OF ETHYL ACETATE FRACTIONS
(E1F6 & E2F6).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₆</u>				
1	1000.0	30	1	>1000
2	100.0	30	0	
3	10.0	30	0	
<u>E₂F₆</u>				
1	1000.0	30	4	>1000
2	100.0	30	2	
3	10.0	30	0	

TABLE-13: DATA OF BIOASSAY OF n-BUTANOL FRACTIONS
(E1F7 & E2F7).

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
<u>E₁F₇</u>				
1	1000.0	30	5	>1000
2	100.0	30	3	
3	10.0	30	1	
<u>E₂F₇</u>				
1	1000.0	30	8	>1000
2	100.0	30	5	
3	10.0	30	3	

TABLE-14: DATA OF SAPONINIC CONTENT EXTRACTED FROM 60gm
EACH OF DRIED LEAVES OF *HEDERA HELIX* COLLECT
FROM DIFFERENT PLACES OF N-W.F.P.

S.No	LOCALITY	HABIT	Wt. OF SAPONIN (gm)	PERCENTAGE (%)
1	Baragali	Climber	2.98	4.97
2	//	creeper	2.82	4.70
3	Marghuzar	Climber	2.25	3.75
4	//	creeper	2.70	4.50
5	Maindam	Climber	2.22	3.70
6	//	creeper	2.04	3.41
7	Abbottabad	Climber	2.50	3.16
8	Peshawar	Climber	2.44	4.07

TABLE-15: DATA OF BIOASSAY OF SAPONIN ISOLATED FROM
DRIED LEAVES OF *HEDERA HELIX* COLLECTED FROM
DIFFERENT PLACES OF N-W.F.P.

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No.of Brine shrimp killed	LC ₅₀ (μg)
1.Baragali (Climber)	1000.0	30	6	>1000
	100.0	30	6	
	10.0	30	5	
2.Baragali (creeper)	1000.0	30	8	>1000
	100.0	30	5	
	10.0	30	4	
3.Marghuzar (Climber)	1000.0	30	7	>1000
	100.0	30	2	
	10.0	30	1	
4.Marghuzar (creeper)	1000.0	30	5	>1000
	100.0	30	5	
	10.0	30	1	
5.Maindam (Climber)	1000.0	30	9	>1000
	100.0	30	3	
	10.0	30	5	
6.Maindam (creeper)	1000.0	30	6	>1000
	100.0	30	4	
	10.0	30	2	
7.Abbottabad (Climber)	1000.0	30	8	>1000
	100.0	30	4	
	10.0	30	2	
8.Peshawar (Climber)	1000.0	30	9	>1000
	100.0	30	2	
	10.0	30	1	

TABLE-16: DATA OF BIOASSAY OF THE "RESIDUE"

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
1	1000.0	30	16	700.54
2	100.0	30	9	
3	10.0	30	3	

TABLE-17: DATA OF BIOASSAY OF COLUMN CHROMATOGRAPHIC FRACTIONS OF THE "RESIDUE".

S.No	Dose ($\mu\text{g/ml}$)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (μg)
FRACTION No1				
1	1000.0	30	21	423.6
2	100.0	30	5	
3	10.0	30	2	
FRACTION No2				
1	1000.0	30	11	>1000
2	100.0	30	2	
3	10.0	30	2	
FRACTION No3				
1	1000.0	30	9	>1000
2	100.0	30	4	
3	10.0	30	1	

FRACTION No1 = Chloroform Fraction.

FRACTION No2 = Chloroform:Methanol(1:1) Fraction.

FRACTION No3 = Methanol Fraction.

For the isolation of cytotoxic compound the chloroform fraction was subjected to preparative TLC, with methanol:chloroform:n-Hexane (1:1:2.5) as mobile phase. The Rf values and percent yield of the six compounds (F1-F6) separated on the preparative TLC of the chloroform fraction are given in Table-18.

The six fractions (F1-F6) were tested for their cytotoxic potentials. The bioassay data of these compounds is given in Table-19. From the table it is clearly evident that only compound F4 , with Rf value 0.889, has very significant cytotoxic activity (LC_{50} 161.84 μ g).

In order to know the chemical nature of the compound F4, it was subjected to phytochemical studies, including Ultraviolet and Infrared spectroscopies and chemical tests. The λ_{max} of the compound F4 in chloroform after UV spectroscopy is 280 nm .

TABLE-18: Rf VALUES AND PERCENT YIELD OF COMPOUNDS (F1-F6) OBTAINED AFTER TLC.

S.N o	Compounds	Rf VALUES	PERCENT YIELD
1	F1	0.375	0.24%
2	F2	0.565	0.18%
3	F3	0.813	0.14%
4	F4	0.889	0.16%
5	F5	0.924	0.12%
6	F6	0.965	0.32%

TABLE-19: DATA OF BIOASSAY OF COMPOUNDS (F1-F6) OBTAINED AFTER THE TLC OF THE CHLOROFORM FRACTION

COMPOUNDS	Dose (µg/ml)	No. of Brine shrimp taken	No. of Brine shrimp killed	LC ₅₀ (µg)
F1				
1	1000.0	30	5	>1000
2	100.0	30	3	
3	10.0	30	2	
F2				
1	1000.0	30	5	>1000
2	100.0	30	2	
3	10.0	30	1	
F3				
1	1000.0	30	3	>1000
2	100.0	30	3	
3	10.0	30	1	
F4				
1	1000.0	30	22	<u>161.84</u>
2	100.0	30	14	
3	10.0	30	4	
F5				
1	1000.0	30	15	>1000
2	100.0	30	8	
3	10.0	30	10	
F6				
1	1000.0	30	10	>1000
2	100.0	30	10	
3	10.0	30	7	

Infrared spectra of the compound F4 (fig-9) showed that there is a phenolic stretch at 3600 cm^{-1} and an aromatic (C=C) bending at 1560 cm^{-1} which is a p-substituted aromatic ring (1700 cm^{-1}). The stretch at $2800\text{--}29500\text{ cm}^{-1}$ is either due to CH_3 , CH_2 , CH of the compound or Nujol, or of the OCH_3 , C-H stretch of the compound. where as the bending at 2310 cm^{-1} indicating presence of $\text{C}\equiv\text{C}$ or $\text{C}\equiv\text{N}$.

Ferric chloride colour test gave an intense green colour which also confirmed the phenolic nature of the F4 compound (Horborne, 1973).

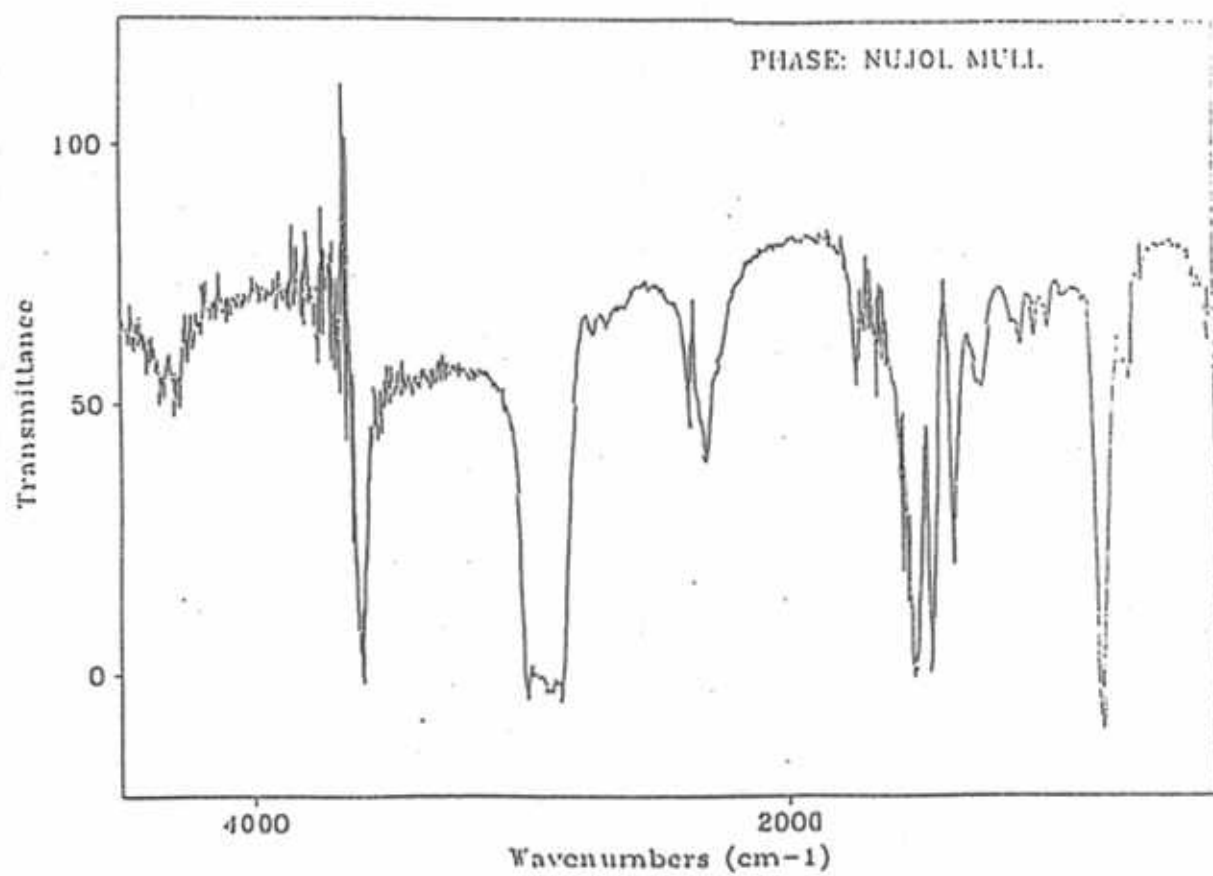


Fig. 8: Infrared Spectra of the Compound **F4**

3.5 ANTIBACTERIAL ACTIVITY

Antibacterial activity of the saponins extracted from the dried leaves of *Hedera helix* (table-20) showed that they are more active against the gram-positive (*Streptococcus facealis*, *Streptococcus pyogenes* & *Streptococcus virigans*) than against the gram-negative ones (*Aeromonas sobriae*, *Vibrio cholera* *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei* & *Proteus vulgaris*) .

TABLE-20: DATA OF THE BACTERIAL SENSITIVITY TOWARD THE
SAPONINS OF *Hedera helix*

Bacteria	Antibacterial Activity
<u>Gram Positive:</u>	
<i>Staphylococcus aureus</i>	-
<i>Streptococcus faecalis</i>	++
<i>Streptococcus pyogenes</i>	++
<i>Streptococcus virigans</i>	++
<i>Bacillus subtilis</i>	+
<i>Corynebacterium diptheriae</i>	+
<u>Gram Negative:</u>	
<i>Aeromonas sobriae</i>	-
<i>Vibrio cholera</i>	+
<i>Escherichia coli</i>	+
<i>Salmonella typhi</i>	-
<i>Shigella sonnei</i>	+
<i>Proteus vulgaris</i>	+

Where + = low, ++ = good, +++ = high and - = no activity.

CHAPTER - IV

DISCUSSION

DISCUSSION

4.1 PHARMACOGNOSTIC STUDY OF *HEDERA HELIX*

4.1.1. Palisade Ratio:

The term palisade ratio was first introduced by Wallis and Dewar (1933). The palisade ratio is an important diagnostic aid in the evaluation of drugs (Wallis, 1985). It is a constant value and did not change with age of the leaf, habitat of the plant and from year to year (Wallis and Forsdike, 1938). The palisade ratio of *Hedera helix* leaf is 1.4. As *Hedera helix* is heterophyllus and can easily be adulterated with leaves of many other plants, therefore palisade ratio would help greatly to identify adulterance in *Hedera helix* leaves.

4.1.2 Stomatal index:

Like palisade ratio, stomatal index is also a diagnostic character of leaves. The average stomatal index

of *Hedera helix* leaf is 10.9 . The stomatal index of most of the medicinally used leaves has been worked out. Trease (1983) has published stomatal index list of some important co-generic medicinal plants. Unlike some other plants e.g. *Atropa belladonna* and *Datura metal*, which have a very wide range of stomatal index (1.7 to 12.2 and 16.4 to 20.4 respectively), the stomatal index (tab-1) of *Hedera helix* leaf has a narrow range (9.6 - 12.5).

4.1.3 Vein islet number:

The vein islet number of *Hedera helix* leaf (Tab-2) ranges from 10.4 - 14.4 with an average of 12.4. Like the other parameters, e.g. the stomatal index and palisade ratio, vein islet number is also a useful diagnostic character of leaf. Trease (1983) has published the vein islet numbers of senna, coca, digitalis and buchu leaves. The mean vein islet number of five morphologically different leaves of *Hedera helix* are 12.2, 14.4, 10.4, and 12.6 respectively. Though there is great morphological variation in *Hedera helix* leaf, but the vein islet number

and other parameters are not affected by the morphological variations.

4.1.4 Powdered drug study of *Hedera helix* leaf

Powder drugs study of the *Hedera helix* leaf (fig. 5) showed that the diagnostic features of the powder are the absences of trichomes, large orbicular stomata, multilayered palisade parenchyma and large amount of rosette aggregates crystals of calcium oxalate.

Most of the crude drugs are used in powder form either for medicinal uses or for their extraction. Unlike whole drugs, powdered drugs are more easily and more frequently adulterated. There are many methods used for the adulteration of various crude drugs. In powdered drugs, generally powdered waste products of suitable colour and density are added e.g., powder olive stone are added to powdered drugs of licorice and pipper and exhausted ginger powder to ginger and red sanders wood is added to chillies (Wallis, 1985).

For the detection of adulteration in powdered drugs, both organoleptic and microscopic evaluations are of great importance. The study of the genuine powder enable us to know its diagnostic features and to detect any adulteration.

4.2 CHEMICAL TESTS

4.2.1. Ash Analysis:

The determination of ash is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter. It is specifically applicable to powdered drugs. A total ash value is useful to exclude drugs, which have been adulterated with chalk, lime or calcium sulphate to either adulterate or improve the appearance of drug (Wallis 1985). The average total ash value of *Hedera helix* leaf, worked out, as per procedure is 8.33%. The total ash consists of an inorganic mixture of metallic salts and silica. In certain drugs unwanted parts of the drug, if not removed, rise the ash value, for example scleroides in the unpeeled pericarp of colocynth and the bark of

glycyrrhiza, which are not required in the powdered drug rises the total ash value (Brain and Turner, 1971).

Hedera helix leaf, like majority of other drugs contain Ca-oxalate. The percentage of calcium oxalate may vary. In some plants like rhubarb, the percentage of ca-oxalate in total ash vary from 8-40 percent (Wallis, 1985), due to which the total ash value become useless. To exclude this variable (Ca-oxalate) from the total ash, acid-insoluble ash value is determined for such drugs, which provide an evidence for the presence or absence of earthy/sandy adulterants in the drug. As *Hedera helix* also contain Ca-oxalate, the acid insoluble was worked out by dissolving the total ash in dilute hydrochloric acid and weighing the dried residue. The average acid insoluble ash of *Hedera helix* is 0.63 percent.

From some drugs active constituents are extracted with solvents. The exhausted material left retain the same shape and, is sometimes used as an adulterant or substitute of the genuine drug. To detect the presence of

material exhausted by water, a water soluble ash value is used (Wallis 1985). The total ash of *Hedera helix* is 8.33% and its water soluble ash value as worked out is 2.07%. To compare, the total and water soluble ash values of exhausted powder of *Hedera helix* leaves were also worked out. The total ash of exhausted powder is 5.86% and its water soluble ash is 0.96%. It is clear from the above figures that the water soluble ash of exhausted material showed a much greater reduction as compared to the unexhausted drug. Wallis (1985) showed that the total ash value of genuine ginger powder is 2.5-6% and water soluble ash is 1.9-3%. The corresponding values for the exhausted ginger are 2-4% and 0.2-0.5% respectively. Similarly these values for genuine tea leaves are 5-7% and 3.2-4.2% respectively while the exhausted tea leaves have 4.4% total ash and 0.7% water soluble ash. This shows that water soluble ash in the case of exhausted material is subject to a much greater reduction than the total ash and therefore, this value can be used as an important indication of the presence of exhausted material, substituted for the genuine drug Wallis (1985).

4.2.2. Detection of Calcium oxalate

Oxalic acid rarely occurs in the free state in the plants, but is extremely common as its calcium salt in the form of crystals, formed as a result of supersaturation of cell sap with it. Ca-oxalate occurs in most plants and in various tissues. These have different shapes in different plants e.g. prisms in senna and hyoscyamus, rosette aggregates in rhubarb, stramonium and clove, single acicular crystals in ipecacuanha and cinnamon, and bundles of acicular crystals as in squill and sandy crystals in belladonna (Esau, 1965).

Calcium oxalate crystals often form a character of considerable diagnostic importance e.g., solanaceous leaves can be distinguished from one another, as belladonna has sandy crystals, stramonium has cluster crystals and henbane has single and twin crystals. Similarly, phytolacca leaves and roots, which both possess acicular crystals, are distinguished from belladonna leaves and roots, which have sandy crystals (Trease & Evans, 1983).

Hedera helix leaves possess calcium oxalate in the

form of rosette aggregates. Many of the mesophyll cells contain these crystals and are quite conspicuous under the microscope. These crystals can also be used as a diagnostic character in the microscopic evaluation of both the whole and powdered dug study of *Hedera helix* leaf.

4.2.3 Screening Test for Triterpenoid Saponin (Licbrman-Burchoral Test) :

Licbrman-Burchoral test (Brain and Turner, 1975) was performed for the detection of triterpenoidal saponins. Red violet colour appeared, showing the presence of triterpenoidal saponins in *Hedera helix* leaves.

4.3 ANTIDIABETIC (HYPOGLYCEMIC) STUDIES OF *HEDERA HELIX*

Through the ages, the plants have been used as source of drugs, administered empirically or otherwise in the cure of disease. During literature survey it was found that about 75 plants, indigenous to the sub-continent have been known to possess hypoglycemic activity (Zafar, 1991),

but their introduction into modern therapy (Allopathic) awaits pharmacological testing by modern methods.

The results (table-3, appendixes 1-4) of aqueous and methanolic extracts of *Hedera helix* leaf equivalent to 4 gm of powdered leaves per Kg body weight clearly showed that both extracts have hypoglycemic effects at a significant level ($P < 0.05$). It can be concluded from the results that the hypoglycemic principle(s) in *Hedera helix* is extractable both in water and methanol, but as compared to methanolic extract the aqueous extract was more effective as it started hypoglycemic effect at 4 hour interval after the oral administration while the methanolic extract started its effect at 8 hour time interval. The hypoglycemic effect of the aqueous extract remained highly significant ($P < 0.005$) upto 12 hour time interval while that of methanolic extract is less significant ($P < 0.05$) during the same time interval.

The difference in the hypoglycemic effect of aqueous and methanolic extracts of *Hedera helix*, based

on statistical significance (Table-3 & -4), might be due to the extraction of inactive fatty materials by the organic solvent, methanol. These fatty materials along with colouring material in the *Hedera helix* leaves comprises about half of the crude methanolic extract. Whereas these fatty materials are not extractable in water, this might leads to dose differences of the two extracts.

As compared to hypoglycemic drug acetohexamide, which started its effect quickly after 2 hour time interval (highly significant), the hypoglycemic effects of the extracts started their effect latter (after 4 hour time interval) but remained significant ($P < 0.005$) up to 12 hour time interval, showing that the crude extract took more time for absorption and onset of action as compared to the finished and properly formulated drugs which are manipulated during manufacturing to obtained desired results regarding their time of absorption and onset of action.

The data obtained after the administration of aqueous and methanolic extracts of *Hedera helix* leaves to alloxan-induced diabetic rabbits, along with an oral hypoglycemic drug, metformin (table-4, appendixes 5-7) shows that both the extracts possess hypoglycemic effect in the alloxan-induced diabetes as well. The hypoglycemic effect of both the aqueous and methanolic extracts started at 8 hour time interval and sustained upto 12 hour time interval, while that of metformin started at 2 hour time interval and sustained upto 12 hours time interval. The hypoglycemic effect of aqueous extract is highly significant ($p < 0.005$) as compared to the methanolic extract ($P < 0.05$) at 8 and 12 hours time intervals. As compared to metformin which started its action at 2 hour time interval and remained upto 12 hour time interval, the aqueous extract started its action at 8 hour time interval but it was as significant as metformin, indicating that the aqueous extract of *Hedera helix* leaves, with proper dose adjustment could be used as effectively as a finished hypoglycemic drugs. The overall comparison is given in fig. 8.

The main constituent of this plant is saponin as we have done most of the work with crude extracts and no such published literature is available on the antidiabetic activity of saponins, therefore, at this stage the correlation of hypoglycemic activity of *Hedera helix* with its saponin constituents seems to be not possible.

Another possibility, that seems to be quite reasonable, is the presence of sufficient amounts of trace elements, called as "HYPOGLYCEMIC ELEMENTS" (Donsbach, 1982), in leaves of *Hedera helix* (table-5). These trace elements are Chromium(Cr^{+++}), Zinc(Zn^{++}) and Manganese(Mn^{++}).

As mentioned earlier, chromium is a vital component of an organo-chromium complex called Glucose Tolerance Factor (GTF). This complex potentiate the action of insulin through facilitating the attachment of insulin to cell membranes, that ultimately enhance the uptake of glucose by the cells (Robinson et al., 1985, Uusitupa et

al., 1983). The deficiency of this complex either through absolute dietary lack of Chromium or reduced ability to synthesize this specific complex, results in glucose intolerance, and reduced efficiency of insulin. symptomatic Chromium deficiency has been clearly demonstrated in man during prolonged intravenous feeding when purified nutrients (Amino acids and Carbohydrates) do not contain sufficient Chromium to meet its requirement.

In two studies, supplementation of intravenous fluid with 250 mg Chromium per day or intravenous supplementation with 150 mg Chromium per day (Ottaway et al., 1986) reversed severe glucose intolerance, improved nitrogen balance and weight gains.

Herber and Gershoff (1973), Kirchgessner et al. (1976) and Wolman (1979) reported that Zinc(Zn) depletion lead to abnormalities in glucose utilization.

Donsbach (1982) reported the correction of glucose tolerance curve with Manganese(Mn) adequate diet.

Keeping all these reports in view it could be suggested that the hypoglycemic activity of the *Hedera helix* might be due to the availability of these "Hypoglycemic Elements" to certain enzymatic processes which have direct or indirect effect on glucose metabolism.

Burger (1960) reported that one of the possible diabetogenic mechanism of alloxan is the removal of zinc from insulin in the form of chelate. So there is a possibility that extract of *Hedera helix* provides the depleted zinc to the insulin which in turn becomes active and produces its normal physiological functions.

Similarly the availability of the chromium and Manganese might have initiated certain enzymatic reactions which in turn affected the glucose metabolism.

4.4 PHYTOCHEMICAL AND CYTOTOXIC STUDIES OF *HEDERA HELIX*

The saponin isolated from the leaves of *Hedera helix* (both Climbers and creepers) collected from different places of N-W.F.P (table-14) showed no significant quantitative or qualitative change in the saponin contents and that there is no effect of climate and height on the saponinic content of *Hedera helix*.

Baragali, Maindam and Marghuzar receive heavy snow fall in the winter months and there is lot of rains in these places. Winter is severe where as summer season is mild. In contrast, Peshawar is comparatively a dry place, with little rains and no snow fall at all. The summer is intensely hot. In spite of these climatic differences, the Saponin contents are not affected and a plant growing in Peshawar contains almost the same amount of Saponin as those present in plants growing in Baragali, Maindam and Marghuzar. This suggests that Saponin production being genetically controlled are least affected by climatic differences.

The result of bioassays of the two methanolic extracts of the dried powdered leaves of *Hedera helix* (E1 & E2) and their different fractions (E1F1-E1F7 & E2F1-E2F7 respectively) showed that only the two methanolic extracts (E1 & E2) and the chloroform fraction of E2, i.e. E2F1 exhibited some what significant cytotoxicity (Table-6 & 7), where as rest of the fractions of both the extracts were found to be inactive (table-8 to 13) as their $LC_{50} > 1000 \mu g$.

The Saponin isolated from plants of various places of N-W.F.P., when assayed also did not show cytotoxicity (table-15). It can be concluded from the above facts that though methanolic extracts of *Hedera helix* leaf are cytotoxic but the saponin isolated are not cytotoxic ($LC_{50} > 1000 \mu g$). This fact is also confirmed by findings of Quertine et al. (1992) that the crude extract of *Hedera helix* exerted cytotoxic activity, both in vitro and in vivo, but the saponin isolated from this plant have no cytotoxic effect on cancer cells.

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The cytotoxic activity of chloroform fraction E2F1 (LC_{50} 700.24 μ g) and methanolic extract E2 (LC_{50} 802.73 μ g) are more significant as compared to the methanolic extract E1 (LC_{50} 942.71 μ g). This difference of activity might be due to the presence of a large amount of coloring and fatty material (about 50% of the weight of dried crude methanolic extract) in E1.

As for as the difference in the cytotoxicity of the extract E2 and its chloroform fraction E2F1 is concerned, it can be presumed that there might be a cytotoxic compound/compounds which is/are more soluble in chloroform than methanol during the course of fractionation.

The bioassay result (table-16) of the fraction left after separation of saponin ,i.e., the "RESIDUE", is very significant (LC_{50} 700.54 μ g), indicating that this part of the extract of dried leaves of *Hedera helix* contained the cytotoxic principles.

In order to isolate the cytotoxic principle from this "RESIDUE", it was further fractionated through column chromatography. The fractions were eluted using chloroform, chloroform:methanol(1:1) and methanol solvents.

Bioassay data (table-17) of these three fractions obtained through column chromatography further revealed that the cytotoxic principle is present in the chloroform fraction (LC_{50} 423.59 μ g), where as the other two fractions(2&3) are not cytotoxic, as their LC_{50} are greater than 1000 mg

For the isolation of cytotoxic compound the chloroform fraction was subjected to preparative TLC, which indicates that this fraction contain six compounds (F1-F6). The R_f values and percent yield of these compounds are given in table-18. These six fraction were next tested for their cytotoxic potentials. The bioassay data of these six compounds is given in table-19. From the table it is clearly evident that only compound F4 ,

with Rf value 0.889, has very significant cytotoxic activity (LC_{50} 161.84 μ g).

In order to know the chemical nature of the compound F4, it was subjected to phytochemical studies, including Ultraviolet and Infrared spectroscopies and chemical tests. The λ_{max} (280 nm) of the compound F4 obtained after UV spectroscopy is closely resembling with phenol (λ_{max} 278 nm) and chlorogenic acid (λ_{max} 260 nm) when run with these compounds in chloroform suggesting phenolic nature of the compound. The phenolic nature of the compound (F4) is also supported by Infrared spectra (fig-9) as there is, a phenolic stretch at 3600 cm^{-1} and an aromatic (C=C) bending at 1560 cm^{-1} which is a p-substituted aromatic ring (1700 cm^{-1}). The stretch at $2800\text{-}29500\text{ cm}^{-1}$ is either due to CH_3 , CH_2 , CH of the compound or Nujol, or of the OCH_3 , C-H stretch of the compound. where as the bending at 2310 cm^{-1} indicating presence of $C\equiv C$ or $C\equiv N$.

Ferric chloride colour test gave an intense green colour which also confirmed the phenolic nature of the F4 compound (Harborne, 1973).

The role of phenolic compound in plant is well established (e.g. the lignin as structural material of cell wall; the anthocyanins as flower pigments (Harborne, 1973); the flavonols appear to be important in regulating control of growth in pea plant (Galston, 1969) and their presence in spinach chloroplasts (Oettmeier et al., 1971) has suggested that they may be involved in photosynthesis (Harborne, 1973).

Johnson et al. (1952) have reported that phenolics present in the plants give resistance against infections. Walker et al. (1955) confirmed the chemical antifungal role of two phenolic compound (Catechol and Protocatechuic acid) in the dead outer skin of coloured onion varieties. Farkas et al. (1962) observed that phenols play protective role even prior to the infection. Stoessl (1970) reported that the phenolic

acids (chlorogenic acid and caffeic acid) give resistance against fungal diseases in potatoes, apples, pears, sweet potatoes, carrots, coffee and wheat.

Phenolic compounds have also been reported as phytotoxic allelochemicals. They inhibit germination and growth of various plants (Hattung et al., 1990; Hussain et al., 1991; Hussain et al., 1993). Phenolic compounds are also known to block cell division by destruction of spindle. Corman (1946) noted that a saturated aqueous solution of coumarine block mitosis in onion and lily roots within 2-3 hours. Hussain et al. (1994) noted that *Imperata cylindrica* contain phenolic phytochemicals (caffeic, p-coumaric, p-hydroxybenzoic, syringic, chlorogenic, isochlorogenic, ferulic and vanillic acids) which suppressed the germination and growth of test species (*Brassica campestris*, *Pennisetum americanum* and *Lactuca sativa*) by inhibiting cell division and cell growth. The cytotoxic effect of phenolic compounds may also be due to inhibition of protein synthesis and change in lipid and organic acid metabolism. For example

Croak (1972) found that coumaric and ferulic acids reduced the incorporation of glucose in to primary protein (alcohol insoluble) and increase the concentration of soluble amino acids. Another possibility of cytotoxic mechanism of phenolic compounds is the inhibition of respiration. Koeppe (1972) noted that 5-hydroxy nepthoquinone, isolated from Juglans species inhibited oxygen up take by excised corn root by more than 90% after one hour treatment. Similarly Van Sumwere et al. (1971) have reported that quinones have inhibitory effect on the oxygen uptake by *Saccharomyces crevisiae*. Phenolic compounds also inhibit the action of specific enzymes. William (1963) noted that simple phenols and oxidized chlorogenic acid, present in the apple juice inhibited the pectolytic enzymes of *Sclerotina frutigena*. Thomas et al. (1966) also observed that phenolic compounds (p-hydroxybenzoic, p-coumaric, vanillic, ferulic and syringic acids) which are present in many soils, when applied to plants growing in nutrient culture solutions were found to suppress the growth of young wheat, corn and soybean. Hussain et al.

(1989) found that *Phalaris minor* contain phenolic allelochemicals (caffeic, ferulic, p-coumeric, chlorogenic, and p-hydroxybenzioc acids) which invariably reduced the seedling growth of wheat, maize, bajra, and kangri and the grain yield in wheat.

Because of the phytotoxic nature, the phenolic compounds generally exist as glycosides in the healthy Plant (Towers, 1964), but are hydrolyzed into phytotoxic phenolic compounds on cell-breakdown by fungal exoenzymes and by mechanical damage, as in crushing the plant. Free phenols are also produced as a consequence of the increased biosynthesis of aromatics following infection. The phenols themselves are usually only weakly phytotoxic but subsequent oxidation furnish quinones which may participate in the resistance process by condensation with proteins and enzymes or by polymerization to tannins and lignins which may act as protein precipitants or as physical barriers to parasite expansion (Stoessl, 1969).

From early 1990s the biological activity, specially the anticancer activity of phenolic compounds of plant origin gained considerable attention, and lot of work has been done in this field so far.

The current knowledge of anticancer activity of Lignins, Quinones and other aromatic compounds has been reviewed excellently by Pettit et al (1994).

A number of phenolic compounds from the higher plants, marine algae, and fungi have been found to possess antitumor and cytotoxic activity. e.g. Phenolic compounds (kneglomeratanol, Kneglomeratanones A and B) isolated from *Knema glomerata* possess cytotoxic activity when tested by brineshrimp bioassay and also showed moderate but significant toxicities against three human tumor cell lines, and inhibited the growth of crown gall tumors on discs of potato tubers (Zang et al., 1994). Some of the polyphenols isolated from marine algae are also reported to possess cytotoxic activity (Blunden et al. (1994). Similarly four phenolic compounds of fungal

origin, Russuphelin A, B, C and D, are reported to exhibit cytotoxic activity against 10 tumor cell lines including human derived carcinoma cells. These compounds are also tested *in vitro* against P388 leukemia cells and are found to be significantly active (Takahashi et al., 1992 and 1993).

Keeping in view the reported cytotoxicity of phenolic compounds and the cytotoxicity of the phenolic compounds isolated from the *Hedera helix*, this plant can be used as one of the natural potential sources of anticancer drug.

Phenols have many useful medicinal uses . It is a bacteriostatic, bactericidal and is also active against certain viruses. In combination with other compounds, it is used as a paint in the inflammatory condition of mouth and throat and in the infection of middle and external ears. The property of precipitating protein by phenols have been practically utilized in curing of

haemorrhoids and piles, enlarged prostate and Hydrocele and epididymal cyst (Martindale, 1982).

4.5 ANTIBACTERIAL ACTIVITY

The preliminary antibacterial screening test of the saponins extracted from the dried leaves of *Hedera helix* (table-20) showed that these are more active against the gram-positive (*Streptococcus faecalis*, *Streptococcus pyogenes* & *Streptococcus virigans*) than against the gram-negative ones (*Aeromonas sobriae*, *Vibrio cholera* *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei* & *Proteus vulgaris*). These results are in conformity with the findings of Cioaca (1978), who performed a microbiological test with 22 strains of both the gram-positive and gram-negative bacteria and found that the saponin of *Hedera helix* are more active against gram-positive bacteria.

As a whole it is evident from the data obtained that the Saponins of *Hedera helix* have a great range of

antibacterial activity and there is great scope for further research work in this respect.

CONCLUSIONS

Hedera helix posses hypoglycemic activity and the study showed that it might be due to the presence of adequate amount of "Hypoglycemic Trace Elements" i.e., chromium, manganese and zinc.

It contains a cytotoxic principle and the phytochemical analysis revealed that it consists of a phenolic nucleus with an unsaturated side chain at para position.

Moreover it also possess antibacterial activity specially against gram-positive bacteria.

SUGGESTIONS FOR FURTHER WORK

Keeping in view the importance of *Hedera helix* the following suggestions are proposed for further research

1. Propagation studies on *Hedera helix*, should be carried out for its mass production.
2. Mass and NMR spectroscopic studies of the cytotoxic principle in order to know it's exact chemical structure.
3. Detailed pharmacological study of the cytotoxic principle both *in vitro* and *in vivo* in animal and human cancer cell lines.

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APPENDIX-1

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF NORMAL RABBITS, GROUP-I,
(CONTROL) RECEIVED 2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at different time intervals (hrs)					
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	93.00	94.00	93.00	92.00	90.00	90.00
R2	94.00	93.00	93.00	91.00	91.00	90.00
R3	93.00	95.00	98.00	91.00	91.00	91.00
R4	94.00	92.00	93.00	92.00	92.00	91.00
R5	97.00	96.00	95.00	93.00	93.00	93.00
R6	94.00	94.00	94.00	93.00	93.00	93.00
MEAN	94.17	94.00	94.33	93.50	91.67	91.33
S.D	1.47	1.41	1.96	1.64	1.21	1.36
SEM	0.60	0.58	0.81	0.67	0.50	0.56

APPENDIX-2

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF NORMAL RABBITS (GROUP-IIa) RECEIVED DOSE OF AQUEOUS EXTRACT EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWERED LEAVES OF *HEDERA HELIX* IN 2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at different time intervals (hrs)					
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	94.00	91.00	90.00	82.00	84.00	93.00
R2	91.00	88.00	88.00	80.00	82.00	90.00
R3	93.00	92.00	84.00	82.00	80.00	95.00
R4	92.00	90.00	90.00	86.00	88.00	90.00
R5	90.00	90.00	89.00	82.00	84.00	92.00
R6	92.00	89.00	89.00	84.00	82.00	94.00
MEAN	92.00	90.00	88.33	82.66	83.33	92.33
S.D	1.41	1.41	2.25	2.06	2.73	2.06
SEM	0.57	0.57	0.92	0.84	1.12	0.84

APPENDIX-3

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF NORMAL RABBITS (GROUP-IIb) RECEIVED DOSE OF METHANOLIC EXTRACT EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWDERED LEAVES OF *Hedera helix* IN 2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at different time intervals (hrs)					
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	89.00	89.00	90.00	86.00	90.00	92.00
R2	94.00	92.00	92.00	89.00	91.00	93.00
R3	91.00	91.00	93.00	90.00	92.00	95.00
R4	93.00	92.00	90.00	88.00	91.00	91.00
R5	94.00	92.00	91.00	89.00	90.00	95.00
R6	90.00	90.00	89.00	85.00	90.00	92.00
MEAN	91.83	91.00	90.83	87.83	90.66	93.00
S.D	2.13	1.26	1.47	1.94	0.82	1.67
SEM	0.87	0.51	0.80	0.79	0.33	0.68

APPENDIX-4

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF NORMAL RABBITS (GROUP-IIc)
RECEIVED DOSE OF ACETOHEXAMIDE, 500 mg/Kg BODY WEIGHT IN 2% GUM
TRAGACANTH SOLUTION.

	BGL (mg/dl) at different time intervals (hrs)					
S.No	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	91.00	78.00	73.00	80.00	85.00	92.00
R2	93.00	83.00	80.00	84.00	89.00	94.00
R3	95.00	84.00	82.00	83.00	89.00	92.00
R4	93.00	81.00	78.00	81.00	87.00	93.00
R5	92.00	80.00	76.00	79.00	85.00	91.00
R6	89.00	72.00	70.00	79.00	88.00	90.00
MEAN	92.16	79.66	76.50	81.00	87.16	92.00
S.D	2.04	4.32	4.46	2.10	1.83	1.41
SEM	0.83	1.76	1.82	0.85	0.75	0.57

APPENDIX-5

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF ALLOXAN-DIABETIC RABBITS (GROUP-IIIa) RECEIVED DOSE OF AQUEOUS EXTRACT EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWDERED LEAVES OF *Hedera helix* IN 2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at different time intervals (hrs)					
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	250.00	248.00	246.00	237.00	235.00	260.00
R2	262.00	260.00	258.00	244.00	242.00	258.00
R3	250.00	250.00	247.00	245.00	240.00	245.00
R4	256.00	254.00	250.00	247.00	245.00	265.00
R5	270.00	265.00	259.00	253.00	250.00	285.00
R6	255.00	253.00	250.00	248.00	245.00	262.00
MEAN	257.17	255.00	251.66	245.66	242.80	262.50
S.D	7.70	6.38	5.53	5.28	5.11	13.00
SEM	3.14	2.61	2.26	2.15	2.09	5.33

APPENDIX-6

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF ALLOXAN-DIABETIC RABBITS (GROUP-IIIb) RECEIVED DOSE OF METHANOLIC EXTRACT EQUIVALENT TO 4gm/Kg BODY WEIGHT OF POWDERED LEAVES OF *Hedera helix* IN 2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at different time intervals (hrs)					
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs
R1	265.00	264.00	265.00	259.00	260.00	255.00
R2	270.00	269.00	268.00	262.00	262.00	265.00
R3	256.00	260.00	258.00	249.00	251.00	270.00
R4	262.00	259.00	260.00	251.00	253.00	265.00
R5	250.00	250.00	248.00	240.00	249.00	255.00
R6	260.00	258.00	255.00	248.00	250.00	262.00
MEAN	260.50	260.00	259.00	251.50	254.16	262.00
S.D	6.98	6.36	7.15	7.96	5.49	6.00
SEM	2.86	2.59	2.92	3.25	2.24	2.45

APPENDIX-7

DATA OF BLOOD GLUCOSE LEVEL (BGL) OF ALLOXAN-DIABETIC RABBITS
(GROUP-IIIc) RECEIVED DOSE OF METFORMIN, 500 mg/Kg BODY WEIGHT IN
2% GUM TRAGACANTH SOLUTION.

S.No	BGL (mg/dl) at		different		time intervals		(hrs)
	0 hrs	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs	
R1	265.00	264.00	265.00	259.00	260.00	255.00	
R2	270.00	269.00	268.00	262.00	262.00	265.00	
R3	256.00	260.00	258.00	249.00	251.00	270.00	
R4	262.00	259.00	260.00	251.00	253.00	265.00	
R5	250.00	250.00	248.00	240.00	249.00	255.00	
R6	260.00	258.00	255.00	248.00	250.00	262.00	
MEAN	260.50	260.00	259.00	251.50	254.16	262.00	
S.D	6.98	6.36	7.15	7.96	5.49	6.00	
SEM	2.86	2.59	2.92	3.25	2.24	2.45	