EFFECT OF ALTERATIONS IN GROWTH TEMPERATURES ON GLYCOTIC REGULATORY ENZYMES OF *Picrorhiza kurroa*

BY-

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UNDER-

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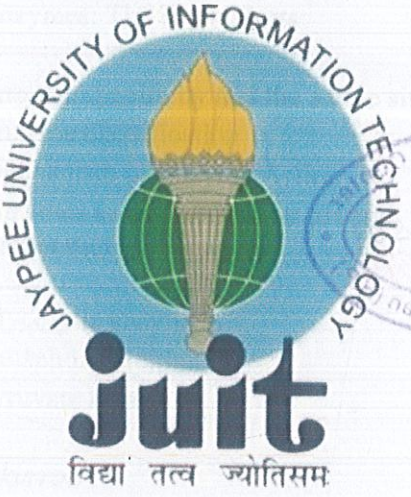

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CERTIFICATE

This is to certify that the work entitled, "**Effect of alterations in growth temperatures on Glycolytic regulatory enzymes of *P. kurroa***" submitted by **Vandana Sharma (081567)** in partial fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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SUMMARY

Picrosides I and II are the most important secondary metabolites present in *Picrorhiza kurroa*. Previous studies have shown that Picroside I and II accumulation was highest in shoots formed at 15^0C at 25^0C. It is very evident that temperature plays a major role in regulating the biosynthesis of Picrosides in *P.kurroa*. Understanding the effect of temperature on the metabolic pathways can lead to optimization of conditions for maximum accumulation of Picrosides in *P.kurroa*.

The aim was to find out the effect of alterations in growth temperatures on the activity of glycolytic regulatory enzymes of *P.kurroa*. The present study was undertaken to establish a link between glycolysis and the secondary metabolite pathway leading to the synthesis of Picrosides. Three *P.kurroa* plant samples i.e. field grown, 15^0C, 25^0C were tested for Phosphofructokinase and Pyruvate kinase activities Maximum specific activity of PFK and PK was observed in 15^0C sample which is well correlated with the highest picrosides content.

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LIST OF ABBREVIATIONS

BSA	-	Bovine Serum Albumin
FC	-	Folin Ciocalteau
O.D.	-	Optical Density
dH_2O	-	distilled water
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAD^+	-	Nicotinamide adenine dinucleotide (oxidized form)
LDH	-	Lactate Dehydrogenase
PFK	-	Phosphofructokinase
PK	-	Pyruvate kinase
TPI	-	Triosephosphate isomerase
ADP	-	Adenosine diphosphate
PEP	-	Phosphoenolpyruvic acid/ phosphoenolpyruvate
F_6P	-	Fructose 6-phosphate
ATP	-	Adenosine diphosphate
P. kurroa	-	*Picrorrhiza kurroa*

CHAPTER 1

INTRODUCTION

1.1 Enzymes: The biocatalysts

Enzymes are proteins with specific catalytic functions produced by all living cells. They are effective in small amounts, remain unchanged after the reaction and do not affect the position of equilibrium of a reversible reaction. They simply increase the speed at which equilibrium is achieved.

Compared with inorganic catalysts, enzymes are very efficient. Enzymes do not require the extremes of temperature and pressure often associated with inorganic catalysts.

Most enzymes are much larger molecules than the substrates they act on, and only a small portion of the enzyme called the active site comes into direct contact with the substrate. The rest of the enzyme molecule functions to maintain the correct shape of the active site. [1]

1.1.1 Enzyme specificity and the active site

Enzymes are extremely specific in their action. The active site of an enzyme typically consists of 3-12 amino acid residues organized into a precise three-dimensional arrangement in a pocket or crevice in the protein. This site has a strong affinity for the substrate because the chemical nature of these amino acid residues and their three-dimensional arrangement form a region that complements certain groupings on the substrate molecule. Examples of the reactive groups of protein side chains that participate in the active site include the thiol group of cysteine, the imidazole group of histidine and the hydroxyl group of serine.

The degree of specificity shown by enzymes for their substrates varies considerably, but usually there is discrimination between stereoisomers. For example, the fungal enzyme glucose oxidase will catalyze the oxidation of D-glucose but not L-glucose to gluconic acid. It even distinguishes between α and β forms:

The rate of β -D-glucose oxidation is about 157 times faster than that for α-D-glucose. Most intracellular enzymes only work on one particular substrate although certain enzymes work on a range of related compounds. An example is an isomerase from Streptomyces or Bacillus species. It is a xylose isomerase, but can also convert glucose into fructose. Glucose and xylose have similar structures, but the former is a hexose and the latter a pentose. [1]

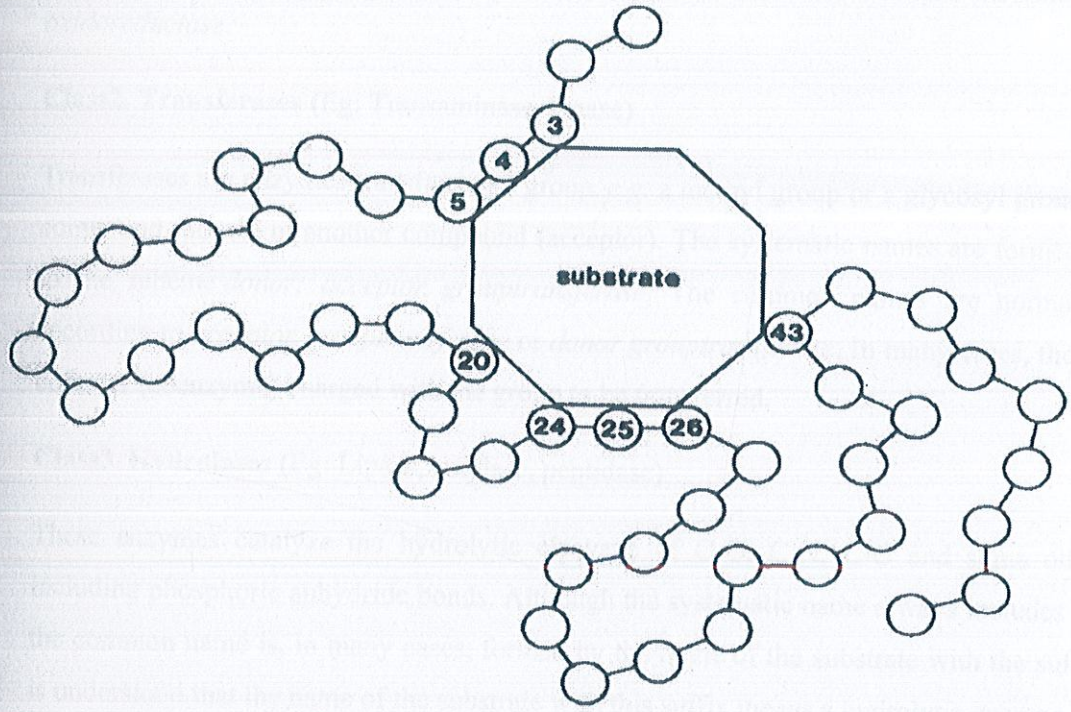


Figure1: *Diagrammatic representation of substrate complexing with an enzyme active site*

1.1.2 Enzyme classification: [2]

The first Enzyme Commission, in its report in 1961, devised a system for classification of enzymes that also serves as a basis for assigning code numbers to them. These code numbers, prefixed by EC, which are now widely in use, contain four elements separated by points, with the following meaning:

(i) the first number shows to which of the six main divisions (classes) the enzyme belongs.

(ii) the second figure indicates the subclass.

(iii) the third figure gives the sub-subclass.

(iv) the fourth figure is the serial number of the enzyme in its sub-subclass.

The main divisions and subclasses are:

Class1. Oxidoreductases (Eg: dehydrogenases, oxidases, reductases)

To this class belong all enzymes catalyzing oxidation/reduction reactions. The substrate that is oxidized is regarded as hydrogen donor. The systematic name is based on *donor: acceptor oxidoreductase*.

Class2. Transferases (Eg: Transaminase, kinase)

Transferases are enzymes transferring a group, *e.g.* a methyl group or a glycosyl group, from one compound (donor) to another compound (acceptor). The systematic names are formed according to the scheme *donor: acceptor grouptransferase*. The common names are normally formed according to *acceptor grouptransferase* or *donor grouptransferase*. In many cases, the donor is a cofactor (coenzyme) charged with the group to be transferred.

Class3. Hydrolases (Eg: Lipase, amylase, peptidase)

These enzymes catalyze the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Although the systematic name always includes *hydrolase*, the common name is, in many cases, formed by the name of the substrate with the suffix *-ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

Class4. Lyases

Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. The systematic name is formed according to the pattern *substrate group-lyase*. In the common names, expressions like *decarboxylase, aldolase, dehydratase* (in case of elimination of CO_2, aldehyde, or water) are used. In cases where the reverse reaction is much more important, or the only one demonstrated, *synthase* (not synthetase) may be used in the name.

Class5. Isomerases

These enzymes catalyze geometric or structural changes within one molecule. According to the type of isomerism, they may be called *racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases* or *cycloisomerases.*

Class6. Ligases

Ligases are enzymes catalyzing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The systematic names are formed on the system *X: Y ligase (ADP-forming).* In earlier editions of the list the term *synthetase* has been used for the common names. Many authors have been confused by the use of the terms *synthetase* (used only for Group 6) and *synthase* (used throughout the list when it is desired to emphasis the synthetic nature of the reaction). Consequently NC-IUB decided in 1983 to abandon the use of synthetase for common names, and to replace them with names of the type *X-Y ligase.*

1.1.3 Mechanism of Enzyme Action

Enzymes catalyze reactions by forming a complex with their substrates at the active site. The first step involves the enzyme binding the substrate rapidly and reversibly to give an ES complex. The rates of the individual forward and backward reactions are k1 and k2 respectively. Subsequent chemical changes occur at the active site involving the breaking and forming of chemical bonds to form the product from ES complex. The rate is characterized by a rate constant kcat. The temperature and pH should remain constant.

The velocity increases up to a certain point, after which no further increase occurs: this is the maximum velocity, V_{max}. The enzyme is now saturated with substrate, and every molecule of enzyme is performing catalysis at its maximum rate under the conditions of the experiment. [1]

Enzyme kinetic constants:

Depending on the precise mechanism of the enzyme's action, Km is a complex function involving the rate constants kl, k2 and kcat. In experimental terms it is numerically equal to

the concentration of the substrate at which the velocity of the reaction is half of the maximum velocity V_{max}.

Importance of Km:

Km is a useful and fundamental characteristic for an enzyme and a particular substrate. It can be viewed as an index of how easily the enzyme can be saturated by the substrate (i.e. the affinity) under defined conditions of temperature and pH. The smaller the value of Km the more readily the enzyme may be saturated with substrate. If the active site of the enzyme can bind and react with several molecules of a similar structure, i.e. there are a number of potential substrates for the enzyme, then each substrate is likely to have a different Km value.

1.1.4 Enzymes are influenced by pH and temperature

Enzymes being proteins are sensitive to changes in the environment in which they work. A change in either the hydrogen ion concentration (pH) or temperature can profoundly affect the activity of an enzyme. This is very important in the industrial use of enzymes. The pH must be controlled and industrial processes usually try to get the maximum rates of activity by choosing the highest temperature at which the enzyme is active but is not denatured.

Most enzymes have a characteristic pH at which the velocity of the catalyzed reaction is maximal and above and below this optimum pH the velocity declines.

The effects of temperature on the activity of an enzyme are complex and can be considered as two forces acting simultaneously but in opposite directions. As the temperature is raised, the rate increases, but at the same time there is a progressive inactivation (denaturation) of the enzyme protein. This becomes more pronounced as the temperature increases, so that an apparent temperature `optimum' is observed. [1]

1.2 Glycolytic enzymes

1.2.1 Phosphofructokinase: (EC 2.7.1.11)

Phosphofructokinase-1 (PFK-1) is one of the most important regulatory enzyme of glycolysis. It is a transferase and catalyzes the phosphorylation of d-fructose 6-phosphate to d-fructose 1,6-bisphosphate. The enzyme has been extensively studied in a wide spectrum of prokaryotes and

eukaryote. At least three forms of PFK are known that differ with respect to the phosphoryl donor. The classical PFK of mammals, yeast and eubacteria, a key enzyme of glycolysis, is ATP-dependent and subject to extensive allosteric regulation by various metabolites. In plants, various protists, and some prokaryotes, pyrophosphate (PPi)-dependent forms of PFK are known (EC 2.7.1.90). These enzymes share sequence similarity with ATP-dependent PFK (ATP-PFK) and are designated either as PPi-PFK or as pyrophosphate: fructose-6- phosphate 1-phosphotransferase. They differ markedly with respect to their regulatory properties across species. [3]

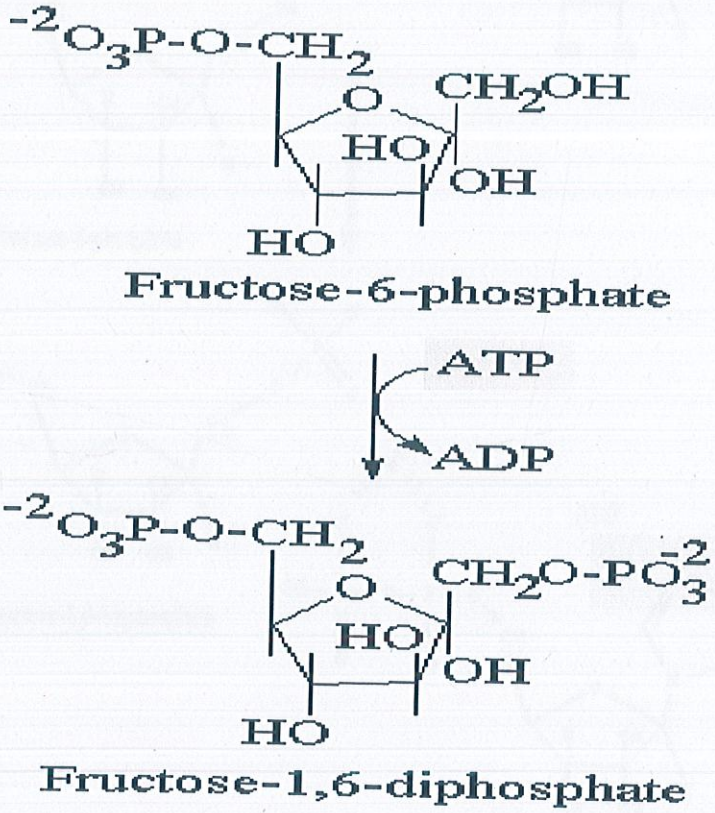


Figure2: *Conversion of Fructose - 6 - phosphate to Fructose-1, 6 - bisphosphate*

PFK reaction is strongly exergonic (irreversible) under physiological conditions and hence is one of the glycolytic pathway's rate-determining steps.

Mechanism of PFK

Phosphofructokinase binds both Mg2+-ATP and fructose-6-phosphate (F6P) to make fructose-1,6-bisphosphate and Mg2+-ADP. [4]

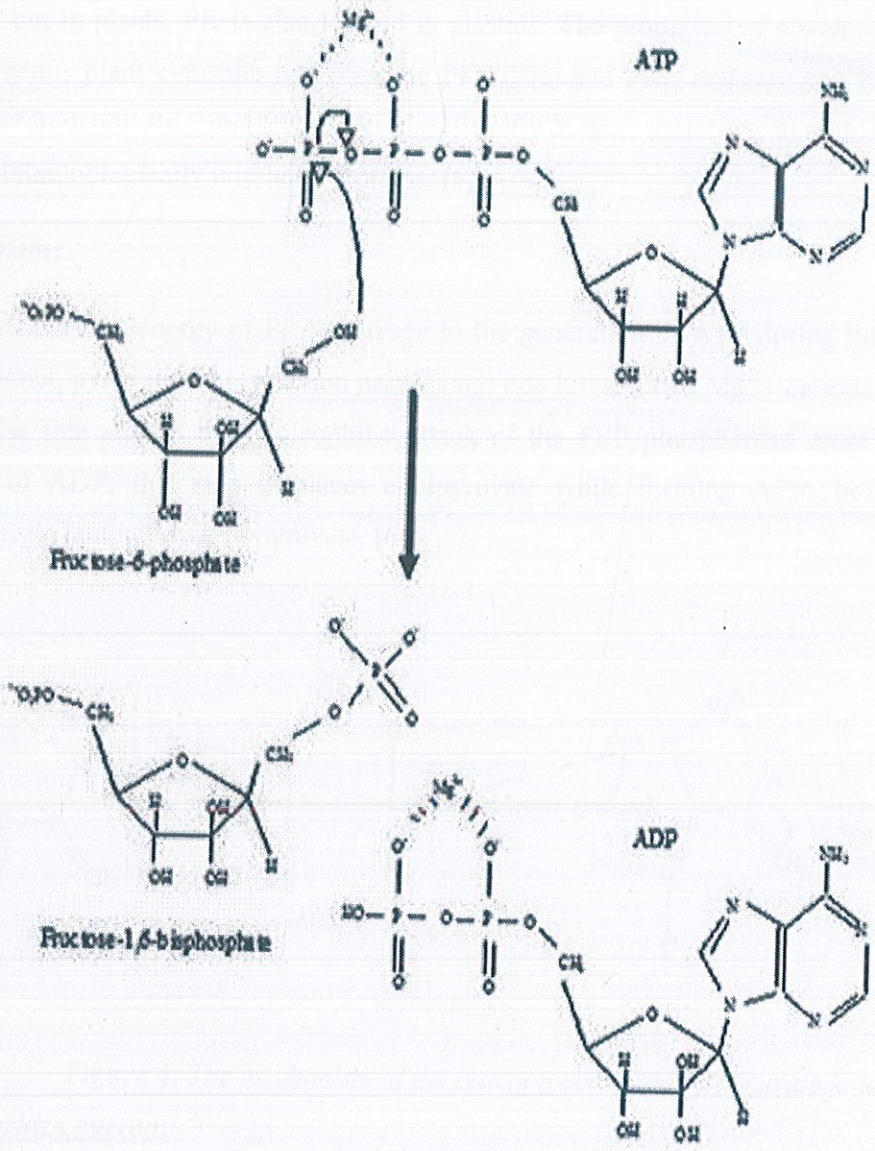


Figure3: *Mechanism of action of PFK*

1.2.2 Pyruvate kinase (EC 2.7.1.40):

PK is a key enzyme of the glycolytic pathway. It catalyzes the irreversible synthesis of pyruvate and ATP from PEP and ADP. It is able to catalyze this step by transferring the phosphate group

from phosphoenolpyruvate (PEP) to ADP. The product, pyruvate, is metabolized by several biosynthetic pathways or is oxidized in the mitochondrion. PK has been isolated and extensively characterized from a wide variety of non-plant sources. In all eukaryotes, PK is found within the cytosol, but in plants, PK is also located in plastids. The properties of several highly purified or homogeneous plant cytosolic and plastidic PKs (PK_c and PK_p, respectively) have recently been examined in detail. PK_c, and PK_p differ significantly in their molecular and kinetic characteristics and are immunologically unrelated proteins. [5]

Mechanism:

It couples the free energy of PEP cleavage to the generation of ATP during the synthesis of the final product, pyruvate. This reaction necessitates one K+ and two Mg2+ cations to be used in two steps. The first step is the nucleophilic attack of the PEP phosphorous atom by β-phosphoryl oxygen of ADP; this step displaces enolpyruvate while forming ATP. In the second step, enolpyruvate tautomerizes to pyruvate. [6]

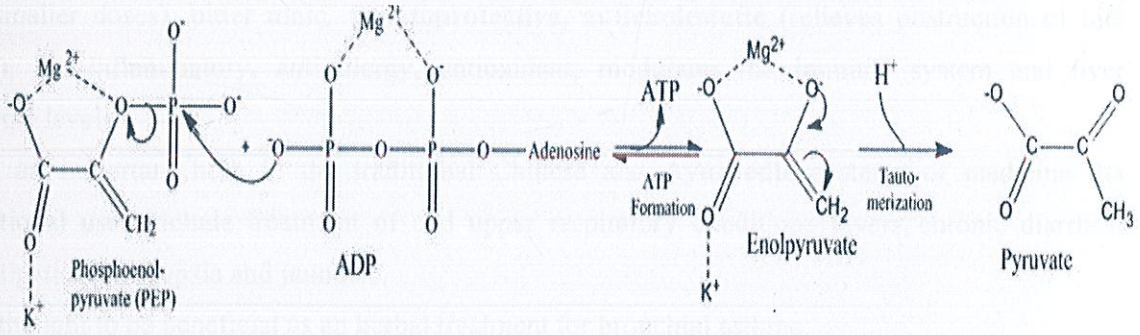


Figure 4: *The mechanism of the reaction catalyzed by pyruvate kinase*

***1.3 Picrorhiza kurroa*:**

Family: Scrophulariaceae

Other Common Names:

Hu huang lian (China), titka kul (Ayurvedic), picrorhiza, kutki, katuka, kutka (Hindu, Sanskrit), kadu (Gujarati).

Habitat:

Picrorhiza kurroa grows in the hilly regions of the North-Western Himalayan region from

Kashmir to Kumaun and Garhwal regions of India and Nepal. It is found at 3,000 – 5,000 meters of elevation.

1.3.1 Plant Description:

The bitter tasting roots of *Picrorhiza kurroa* are hard, about 6-10 inches in length, and creeping. The leaves are 2-4 inches long, oval in shape. The fruit is ½ inch long and oval in shape. The rhizome of *Picrorhiza kurroa* is manually harvested in October through December.

Plant Part Used:

Leaf, bark, root and rhizomes.

1.3.2 Therapeutic Uses: [7]

- *Picrorhiza kurroa* also contains **apocynin**, a powerful anti-inflammatory agent, which also reduces platelet aggregation.
- The actions of *Picrorhiza kurroa* are antibacterial, antiperiodic, cathartic (in large doses), laxative (in smaller doses), bitter tonic, **hepatoprotective, anticholestatic** (relieves obstruction of bile salts), anti-inflammatory, anti-allergy, antioxidant; modulates the immune system and liver enzyme levels.
- It is an important herb in the traditional Chinese and **Ayurvedic** systems of medicine. Its traditional uses include treatment of and upper respiratory conditions fevers, chronic diarrhea, constipation, dyspepsia and jaundice.
- It is thought to be beneficial as an herbal treatment for bronchial asthma.
- It is thought to be helpful as a remedy for a number of **auto-immune diseases** such as vitiligo and psoriasis. In treating viral hepatitis constituents of *Picrorhiza kurroa* may protect against liver damage due to Amanita mushroom poisoning.
- Picrorhiza is a traditional herbal treatment for scorpion stings and snake bites.

Side Effects and Possible Interactions:

Picrorhiza root extracts are widely used in India with no adverse effects being reported. Side-effects and risks are generally less common and serious than in synthetic drugs.

1.3.3 Secondary Metabolites present:

- Picroside I
- Picroside II
- Picroside III
- Picroside IV
- Kutkoside
- Apocynin
- Catechol
- Androsin

Location of secondary metabolite- picrosides

- Shoot – picroside 1
- Rhizome – picroside 1 & 2
- Root – picroside 2

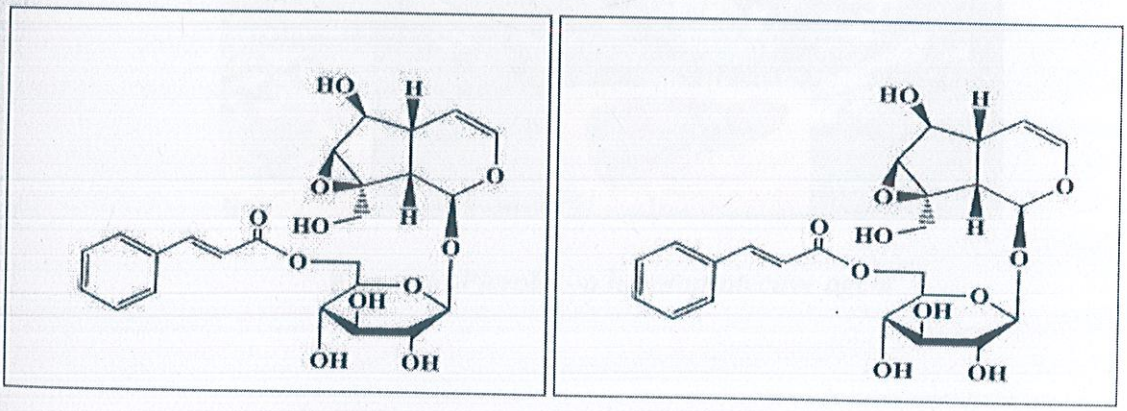


Figure 5: *Structure of picroside I and picroside II*

1.3.4 Drugs:

- **Picroliv** is a hepatoprotective agent of plant origin. It is an iridoid glycoside mixture containing 60% picroside I and kutoside in the ratio of 1:1.5 obtained from the plant, *Picrorhiza kurroa* (root and rhizome).

  Picroliv has shown anti-cholestatic effect in rat, guinea pigs and cats treated with paracetamol and ethinyl estradiol. It has also antiviral and immunostimulant activities. Picroliv is devoid of any significant CNS and CVS, autonomic and other systemic activity. It is developed by CDRI, Lucknow.

  Picroliv appears to be safe in rats and monkeys and has excellent therapeutic index. Phase I and II clinical trials have been conducted and the drug has shown no side effect and is well tolerated.

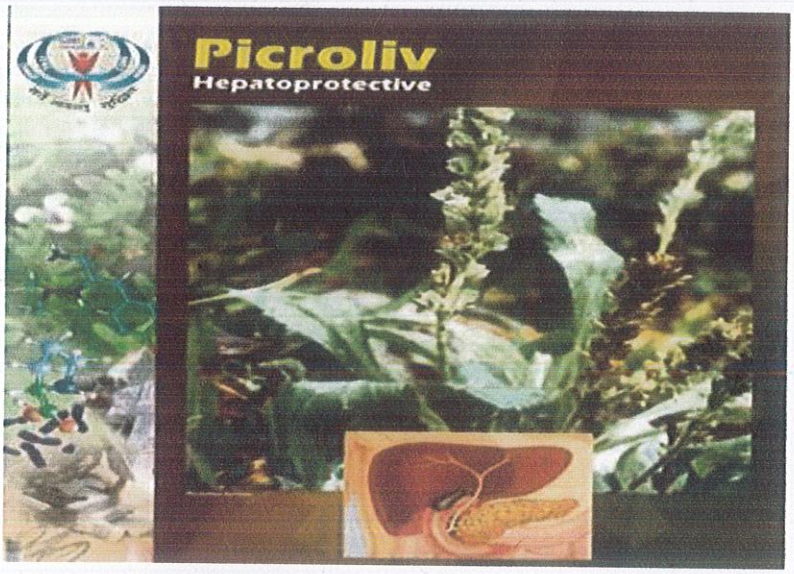


Figure 6: *Picroliv –a hepatoprotective agent*

- **Picrolax** is used for the management of acute constipation. Picrolax is a US patented herbal formulation, proven as a gentle, safe, and effective laxative. It assures smooth evacuation of feces without disturbing the fluid-electrolyte balance.

  Picrolax capsule is not known to have any side effects if taken as per the prescribed dosage.

Picrolax capsule is useful in acute sporadic constipation. It is useful in constipation and is associated with hemorrhoids, in pre- and postoperative conditions, in cardiac patients.

Composition: Each Picrolax capsule contains extracts of Katuka (*Picrorhiza kurroa*) -150mg

Quantity: 10 Capsules

Figure 7: *Picrolax from Himalaya*

CHAPTER 2

LITERATURE REVIEW

2.1 Picrorhiza and its importance

The genus Picrorhiza (Family: Scrophulariaceae) comprises of two species *Picrorhiza kurroa* Royle ex Benth and *Picrorhiza scrophulariiflora* Pennel. *P. Kurrooa* is found in the Western Himalayan regions of Northern India whereas *P. scrophulariiflora* is predominant in the Himalayan regions of Sikkim, Nepal and Tibet (Bantawa et al., 2010, 2011). Though the plant is widely distributed in the Himalayas occurring in Pakistan, India, Nepal, Bhutan and Southern China but their population have declined in some parts of their range owing to overharvest for medicinal use and trade by the traders. [8]

Importance of Picrorhiza:

Both the species are extensively used in traditional medicine system of India, China, Tibet, Nepal and Sri Lanka for the treatment of various types of diseases. Kutki, as *P. kurroa* is commonly known is an important medicinal plant used in traditional as well in modern medicines for the treatment of various immune and liver disorders. It is also useful in gastrointestinal and urinary disorders, leukoderma, snake bite and scorpion stings. It also possesses hepatoprotective and anti-inflammatory effects. It helps in allergies and has free radical scavenging effects in the body. *P. scrofloriiflora* is widely used for the treatment of dysentery, jaundice. Its roots are used as a tonic, cathartic and in improving appetite and digestion. [8]

2.2 Active and inactive plant constituents:

Inactive constituents are those which do not possess any definite therapeutic value .As the formation of different active and inactive constituents of plants involves various metabolic pathways, hence in general the inactive plant constituents are termed as primary plant metabolites whereas active plant constituents are termed as secondary plant metabolites.

Primary metabolites

Primary plant metabolites are simple molecules or polymers of simple molecules synthesized by plants, generally do not possess therapeutic as such but essential for the life of plants and contains high-energy bonds. These are involved in the biosynthesis of secondary metabolites.

Secondary metabolites

Secondary metabolites are complex organic molecules biosynthesized from primary plant metabolites in plant cells .They generally possess therapeutic activity (unique to plants or group of plants) which is neither essential for plants life nor contains high energy bonds. These are usually stored in vacuoles. Secondary metabolites are classified as: alkaloids, glycosides, tannins, phenolic compounds, volatile oils, terpenoids, saponins, steroids, resins and bitter principles. These are used as medicine, food, flavors, colours, dyes, poisons and perfumes etc. [9]

2.3 Active constituents present in *P.kurroa*

The active constituents are obtained from the roots and rhizomes *Picrorhiza kurroa* contains active constituents like picroside-i, picroside-ii, picroside-iii, picroside-iv, apocynin, androsin, catechol, kutkoside, etc. [10]

A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing picroside 1 and kutkoside was launched as a hepatoprotective drug. Picroside-I is the major ingredient of Picroliv and, therefore, makes this compound a highly valued secondary metabolite of P. kurroa. [11]

2.4 Threats to the *P.kurroa* population and possible solutions

Over exploitation and consequent degradation of natural habitat are reported to be a major threat to this plant. Over 90% of the market demand for this species is met from the wild. Due to narrow distribution range, small population size and high use value, the species figure among the 37 identified as top priority species for conservation and cultivation in Western Himalayas. Indiscriminate, unscientific harvesting and lack of organized cultivation of the plant has threatened its status in wild and it is listed as "endangered" species by International Union for Conservation of Nature and Natural Resources (Nayar and Sastri, 1990). [8]

Being pharmacologically important and listed as an endangered herb, *in vitro* regeneration through Plant tissue culture appears as an alternative. This will help in the selection of cell lines with enhanced content of phytopharmaceuticals, in the genetic transformation of *P. kurroa* and most importantly in the production of metabolites in cell cultures. The secondary metabolites can be accumulated in the plant cell cultures in abundant amounts and used for the preparation of various drugs. [10]

Optimization of cell cultures conditions is necessary for large-scale production of metabolites in vitro. However, the production of metabolites through tissue cultures of P. *kurroa* requires thorough understanding of their biosynthesis and accumulation in different morphogenetic tissue culture stages .The biosynthesis and accumulation of Picroside-I and Picroside-II occur in P. kurroa at high altitudes and that too during a particular time of a season, which complicates the process of understanding biology of their biosynthesis. [11]

2.5 Glycolysis

Glycolysis is the "central" metabolic pathway in almost all the organisms. It is a ubiquitous metabolic pathway can show significant differences in terms of its roles, structure, regulation, and compartmentation in different phyla, and even within different cells of the same species. Glycolysis is also directly involved in many biochemical adaptations of plant and non plant species to environmental stresses such as nutrient limitation, osmotic stress, drought, cold/freezing, and anoxia. [12]

Functions of glycolysis:

Glycolysis evolved as a catabolic anaerobic pathway to fulfill two fundamental roles. It oxidizes hexoses to generate ATP, and pyruvate, and it produces building blocks for anabolism. Glycolysis is also an amphibolic pathway because it can function in reverse to generate hexoses from various low molecular-weight compounds in energy-dependent gluconeogenesis. Much attention has been devoted to determining the mechanisms by which the opposing processes of glycolysis and gluconeogenesis are reciprocally regulated in vivo (5). Glycolysis is thus of crucial importance in plants because it is the predominant pathway that "fuels" plant respiration. Also, a significant proportion of the carbon that enters the plant glycolytic and tricarboxylic acid (TCA) cycle

pathways is not oxidized to CO_2 but is utilized in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids. The biosynthetic role of glycolysis and respiration is particularly important in actively growing autotrophic tissues. [12]

Compartmentation of Glycolysis

The sequential conversion of hexoses to pyruvate in plants can occur independently in either of two subcellular compartments, the cytosol and plastid. Compartmentation concentrates enzymes of a pathway and their associated metabolites and prevents the simultaneous occurrence of potentially incompatible metabolic processes .For integration of cellular metabolism, there are controlled interactions between pathways sequestered in various subcellular compartments. Thus, plastidic and cytosolic glycolysis can interact through the action of highly selective transporters present in the inner plastid envelope. [12]

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of sample

Three different samples of *Picrorhiza kurroa* plant (Field grown, 15 ^{0}C, 25 ^{0}C) were collected from the Bio Labs, JUIT. The Field grown sample (2.5 years old) was taken from the Greenhouse. 15 ^{0}C and 25 ^{0}C samples were taken from the Culture rooms, Plant Tissue Culture Lab.

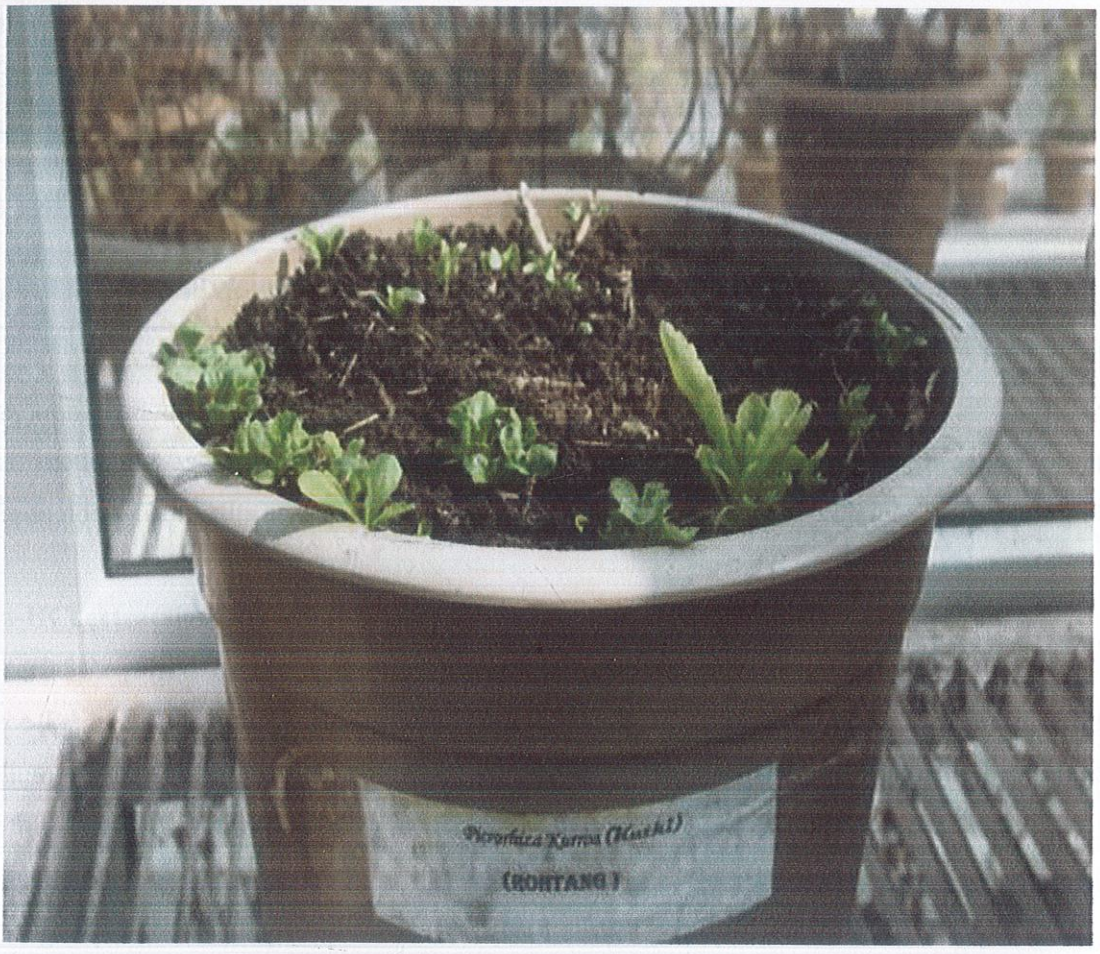


Figure8: *Picture of field grown P.kurroa taken from the greenhouse.*

3.2 Sample Preparation

Materials Required:

P. kurroa samples, liquid nitrogen, pre-chilled pestle and mortar (kept overnight at -80^{0}C), Spatula, surgical blades, gloves.

Procedure:

1. All three plants were washed thoroughly with distilled water and dried.
2. The roots were separated and the shoots and leaves were separated.
3. The shoots and leaves were cut into extremely small pieces with the help of surgical blade in the chilled mortar.
4. The liquid nitrogen was poured onto the small pieces of shoots and leaves.
5. The small pieces were homogenized into fine powder with the help of pestle and mortar.
6. Without allowing the powder to absorb moisture, it was weighed and collected into tarson tubes.
7. The powdered sample of each plant was stored at -80^{0}C.

3.3 Extraction of proteins from *Picrorhiza kurroa* shoots and leaves

Proteins were extracted from the three P.*kurroa* samples (Field grown, 15^{0}C, 25^{0}C) by Tris –HCl extraction method.

This method was particularly favored over other extraction methods because of high reproducibility of results as is confirmed by other scholars working on proteins.

Tris-HCl extraction method

Material Required:

Three P.*kurroa* samples(Field grown, 15^{0}C, 25^{0}C) in the powdered form, Tris – HCl, NaCl, Sodium azide, Centrifuge, -20^{0}C and 4^{0}C refrigerator, magnetic stirrer , magnetic bead, falcons (15 ml), beaker (25 ml), foil and eppendorfs.

Procedure:

1. 0.1 g of powdered sample was taken in a small beaker (25ml).
2. 4 ml of extraction buffer (pH 7.4), containing 50 Mm Tris - HCl, 0.25 M NaCl and 0.01% sodium azide was added to the beaker.
3. The magnetic bead was put in the beaker and it was placed on the magnetic stirrer at 4°C.
4. It was kept overnight with gentle stirring.

5. The mouth of the beaker was covered with the foil.

6. After 24 hrs, the contents of beaker were transferred to 15 ml tubes.

7. The slurry was centrifuged at 10.000 g (approx 12750 rpm) for 20 minutes at 4 C to remove the supernatant was retained.

8. The aliquots of dissolved protein were prepared by transferring 0.5 ml of dissolved protein in 2 ml eppendorfs and stored at -20°C.

9. The entire extraction procedure was carried out for all the three samples.

3.4 Estimation of protein content

Protein content was determined in the three P.*kurroa* samples by the method of *Lowry et al.* (1951).

Principle:

The presence of aromatic amino acids in protein is the basis for the reaction. Proteins react with folin-coilacteu reagent to form a colored complex. The color's intensity depends upon the amount of aromatic amino acids present.

Reagents:

1. Lowry's reagent (D):Mixture of reagents A,B,C in the ratio of 1:1:98(Reagent D was prepared fresh every time).
2. FC reagent (2N Stock): working concentration of folin – ciocalteau reagent - 1N. Dilute it with dH_2O in 1:1 ratio.
3. BSA stock solution – 1mg/ml

Standard working concentrations of BSA –100µg/ml, 300µg/ml, 500µg/ml, 750 µg/ml, 900µg/ml.

Procedure:

1. BSA was diluted (1:10) to the final concentration of 100µg/ml.
2. The experiment was done in triplets.
3. After adding Lowry's reagent, the test tubes (covered with aluminium foil) were vortexed and kept at room temperature for 10 minutes.
4. FC reagent was added; the tubes were vortexed and kept at room temperature for 30 minutes.
5. Finally, the O.D. was taken at 720/670 nm.

BSA Standardization:

BSA(µg/ml)	dH_2O(µl)	ReagentD(ml)	FC(ml)
Blank	1000	4	0.4
100	900	4	0.4
300	700	4	0.4
500	500	4	0.4
700	300	4	0.4
900	100	4	0.4

Table 1: *Different concentrations for BSA standard*

After successful standardization of BSA, 1:1 dilutions (total volume 100 µg/ml) were prepared for all three samples were prepared and protein estimation was done simultaneously by using BSA(100 µg/ml) as a standard.

Determination of Protein Content by Lowry's method:

Concentrations (µg/ml)	dH20(µl)	ReagentD(ml)	FC(ml)
Blank	1000	4	0.4
S1(100)	900	4	0.4
S2(200)	700	4	0.4
P1(100)	500	4	0.4
P2(100)	300	4	0.4
P3(100)	100	4	0.4

Table 2: *Different concentrations for Protein content determination*

S1 (100) = BSA standard1 (concentration is 100 µg/ml)

S2 (200) = BSA standard2 (concentration is 200 µg/ml)

P1 (100) = homogenate of field grown *P.kurroa* sample (Diluted 2 times, concentration used is 100µg/ml)

P2 (100) = homogenate of 15^0Cgrown *P.kurroa* sample (Diluted 2 times, concentration used is 100µg/ml)

P3 (100) = homogenate of 25^0Cgrown *P.kurroa* sample (Diluted 2 times, concentration used is 100µg/ml)

3.5 Phosphofructokinase enzyme assay

Enzyme assays:

NADH-linked Spectrophotometric enzyme assays were done for determining phosphofructokinase activity using the method described by *Simcox et al., 1977.* [13]

Principle (coupled assays):

In spectrophotometric assays, the reaction is followed by measuring the change in the light absorbed by the assay solution.

In an NADH-linked assays, NADH is used as a substrate. As more and more NADH gets

5. Finally, add 0.1 ml of 1:1 diluted 25 ^{0}C sample homogenate was added to 25^{0}C (1) and 25^{0}C(2).
6. Immediately after adding the homogenates, O.D. was taken at 340 nm.

3.6 Puruvate kinase enzyme assay

Enzyme assays:

NADH-linked Spectrophotometric enzyme assays were done for determining phosphofructokinase activity using the method described by *Simcox et al., 1977.* [13]

Principle (coupled assays):

In spectrophotometric assays, the reaction is followed by measuring the change in the light absorbed by the assay solution.

The reaction is catalyzed by lactate dehydrogenase (an oxidoreductase) which uses NADH as a substrate. As more and more NADH gets consumed, UV absorbance (at 340 nm) decreases which can be detected. Absorbance decreases because NADH & NADPH absorb UV light in their reduced forms but not in their oxidized forms (NAD^+).

Assay mixture for pyruvate kinase:

The oxidation of NADH was measured by monitoring the decrease in A at 340 nm.

Component:	**Concentration:**
ADP	0.5 mM
PEP	0.5 mM
$MgCl_2$	10 mM
KCl	45mM
LDH	2 units
NADH	80 u M
Tris-HCL	100mM
Total volume	0.5 ml

Table 4: *Assay mixture for pyruvate kinase*

The assay was done simultaneously for field grown, 15^0C and 25^0C in duplicates. Moreover, the total final volume of the assay mixture was reduced to 1ml from 3ml. Therefore, calculations were done accordingly for the final volume of 8ml (2ml each for Blank, field, 15^0C and 25^0C samples).

Procedure:

1. An assay mixture of volume 8 ml was prepared. Two test tubes were taken and 1ml of assay mixture was poured in each for preparation of Blank.
2. Again, six test tubes were taken and 0.9 ml of assay mixture was poured in each. They were labeled as Field(1)/Field(2), 15^0C(1)/ 15^0C(2) and 25^0C(1)/25^0C(2).
3. 0.1 ml of 1:1 diluted field sample homogenate was added to Field(1) and Field(2) test tubes.
4. Similarly, 0.1 ml of 1:1 diluted 15 ^{0}C sample homogenate was added to 15^0C(1) and 15^0C(2).
5. Finally, add 0.1 ml of 1:1 diluted 25 ^{0}C sample homogenate was added to 25^0C (1) and 25^0C (2)
6. Immediately after adding the homogenates, O.D. was taken at 340 nm.

CHAPTER 4

RESULTS AND ANALYSIS

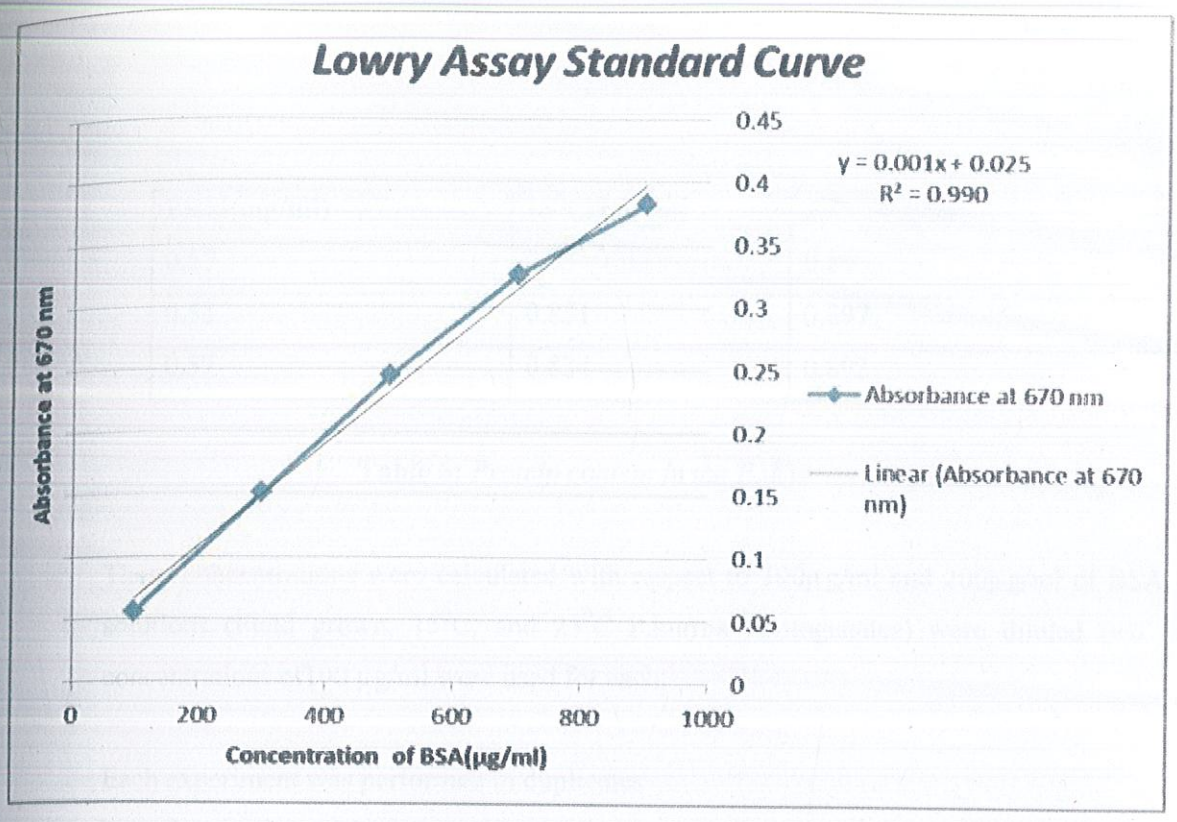
4.1 Determination of protein content in P.kurroa samples using BSA as a standard.

BSA standardization was done and BSA standard curve was plotted initially. Protein estimation was carried out using BSA concentrations of 100µg/ml and 200µg/ml as standards by the method of *Lowry et al.* (1951). BSA was used as a standard because it is pure, stable and inexpensive.

BSA standardization:

Conc(µg/ml)	A1	A2	A3	Average(A_a)
100	0.041	0.072	0.054	0.056
300	0.130	0.156	0.167	0.151
500	0.273	0.234	0.230	0.245
700	0.292	0.339	0.348	0.326
900	0.318	0.424	0.404	0.382

Table 5*: Absorbance readings (670 nm) for different BSA concentrations*



Graph1: *Lowry Assay Standard Curve*

Each experiment was performed in triplicates.

No. of times the experiment was repeated = 5

Determination of Protein content:

Formula used:

Conc. = (O.D. of test * conc.of std. *d.f.)/O.D. of std.

O.D. = Optical density at 670 nm.

Conc.of std. = concentration of standard (µg/ml)

d.f. = dilution factor

Field(mg/ml)	15^{0}C(mg/ml)	25^{0}C(mg/ml)
0.57	0.83	0.89
0.55	0.831	0.897
0.57	0.834	0.892

Table 6: *Protein content in the P. Kurroa samples*

These concentrations were calculated with respect to 100µ g/ml and 200µ g/ml of BSA. The test solutions (Field grown, 15^{0}C, and 25^{0}C P.kurroa homogenates) were diluted two times and concentrations of100 µg/ml were used for each.

Each experiment was performed in duplicates

No. of times the experiment was repeated = 3

Field(mg/ml)	15^{0}C(mg/ml)	25^{0}C(mg/ml)
0.563±0.01	0.831±0.002	0.893±0.003

Table 7: *Average Protein content in P.kurroa samples*

Analysis:

For the three samples, the protein concentration was found to be in the order 25^{0}C sample>15^{0}C sample> Field grown sample.

4.2 Determination of Enzyme activity-Phosphofructokinase assay

The protocol for PFK assay was followed as described by *Simcox et al., 1977.* [13]

Experiment 1

Time (min)	Field		15^{0}C		25^{0}C	
	A1	A2	A3	A4	A5	A6
0	0.487	0.183	0.365	0.374	0.093	0.088
0.5	0.483	0.180	0.361	0.372	0.090	0.085
1	0.483	1.177	0.360	0.359	0.089	0.085
2	0.482	0.177	0.359	0.358	0.087	0.082
3	0.479	0.176	0.356	0.352	0.086	0.080
4	0.478	0.174	0.348	0.350	0.083	0.079

Table 8: *Absorbance readings for Field, 15 ^{0}C and 25 ^{0}C samples at different time intervals*

Experiment2:

Time (min)	Field		15^{0}C		25^{0}C	
0	A1	A2	A3	A4	A5	A6
0.5	0.186	0.184	0.092	0.086	0.058	0.067
1	0.183	0.182	0.091	0.086	0.057	0.065
2	0.182	0.181	0.088	0.080	0.056	0.062
3	0.180	0.178	0.081	0.076	0.050	0.061
4	0.179	0.172	0.075	0.074	0.050	0.060
5	0.178	0.171	0.068	0.068	0.046	0.058

Table 9: *Absorbance readings for Field, 15^{0}C and 25 ^{0}C samples at different time intervals*

Experiment3:

Time (min)	Field		15^0C		25^0C	
0	A1	A2	A3	A4	A5	A6
0.5	0.5400	0.5820	0.108	0.098	0.228	0.316
1	0.5380	0.5800	0.102	0.092	0.224	0.311
2	0.5340	0.5780	0.092	0.090	0.223	0.309
3	0.5310	0.5730	0.088	0.082	0.222	0.307
4	0.5310	0.5720	0.087	0.077	0.219	0.306
5	0.5300	0.5720	0.086	0.074	0.217	0.306

Table 10: *Absorbance readings for Field, 15^0C and 25 ^{0}C samples at different time intervals*

Each experiment was performed in duplicates

No. of times the experiment was repeated = 3

Calculation of $\Delta A_{340/min}$

From the absorbance readings, change in absorbanceΔ(A) was calculated by subtracting the readings from the previous readings.

$\Delta A1 = A_{0.5min} - A_{0min}$

$\Delta A2 = A_{1min} - A_{0.5min}$

$\Delta A3 = A_{2min} - A_{1min}$

$\Delta A4 = A_{3min} - A_{2min}$

$\Delta A5 = A_{4min} - A_{3min}$

	Field(duplicates)		15^0C(duplicates)		25^0C (duplicates)	
ΔA 1	0.004	0.003	0.004	0.002	0.003	0.003
ΔA 2	0.000	0.003	0.001	0.003	0.001	0.000
ΔA 3	0.001	0.000	0.001	0.001	0.002	0.003
ΔA 4	0.003	0.001	0.003	0.006	0.001	0.002
ΔA 5	0.001	0.002	0.008	0.002	0.003	0.001

Table 11: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time intervals*

The same was repeated for Experiment 2 and Experiment 3:

	Field(duplicates)		15 ^{0}C(duplicates)		25 ^{0}C(duplicates)	
ΔA 1	0.003	0.002	0.001	0.000	0.001	0.002
ΔA 2	0.001	0.001	0.003	0.006	0.001	0.003
ΔA 3	0.002	0.003	0.007	0.004	0.006	0.001
ΔA 4	0.001	0.002	0.006	0.002	0.000	0.001
ΔA 5	0.001	0.001	0.007	0.006	0.004	0.002

Table 12: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time intervals*

	Field(duplicates)		15 ^{0}C(duplicates)		25 ^{0}C(duplicates)	
ΔA 1	0.002	0.002	0.006	0.006	0.004	0.005
ΔA 2	0.004	0.002	0.010	0.002	0.001	0.002
ΔA 3	0.003	0.005	0.004	0.008	0.001	0.002
ΔA 4	0.000	0.001	0.001	0.005	0.003	0.001
ΔA 5	0.001	0.000	0.001	0.003	0.002	0.001

Table 13: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time intervals*

Calculation of enzyme activity:

Activity (U/ml) = $\Delta A_{340/min} / \varepsilon_{NADH,340}$

U/ml = units per ml (Units are µmoles/min/ml)

$\Delta A_{340/min}$ = change in absorbance at 340 nm

$\varepsilon_{NADH,340}$ = molar absorption coefficient/ molar extinction coefficient of NADH at 340 nm

$\varepsilon_{NADH,340} = 6.22 * 10^3 M^{-1} cm^{-1}$

$= \Delta A_{340/min} (min^{-1}) / (6.22 * 10^3 M^{-1} cm^{-1})$

$= \Delta A340/min (min^{-1}) / (6.22 * 10^3 moles^{-1} Litre cm^{-1})$

$= \Delta A_{340/min} (min^{-1}) / (6.22 * 10^3 * 10^3 moles^{-1} ml cm^{-1})$

$= (\Delta A_{340/min}/ 6.22) * 10^{-6} moles ml^{-1} min^{-1}$

$= (\Delta A_{340/min}/ 6.22)$ µmoles/min/ml

$= (\Delta A_{340/min}/ 6.22)$ U/ml

Calculation of specific activity:

Specific activity (U/mg) = Activity (U/mg)/Protein content (mg/ml)

U/mg= units per mg (Units are µmoles/min/mg)

Therefore, specific activity = $(\Delta A_{340/min}) * (1/ \varepsilon_{NADH, 340}) *$(1/ Protein content)

Field(U/mg)	15^{0}C(U/mg)	25^{0}C(U/mg)
0.00045	0.00073	0.00036
0.00048	0.00077	0.00039
0.0005	0.00081	0.0004

Table 14: *PFK specific activity (U/mg) in Field, 15 ^{0}C, 25 ^{0}C samples*

	Field(U/mg)	15^{0}C(U/mg)	25^{0}C(U/mg)
Mean	0.00047±0.000025	0.00077±0.00004	0.00038±0.00002

Table 15: *Average PFK specific activity of the three samples*

Analysis:

The PFK specific activity of the three *P.kurroa* plant samples follows the order 15^0C > Field > 25^0C.

PFK specific activity was highest in the 15^0 C plant.

4.3 Determination of Enzyme activity-Pyruvate kinase assay

The protocol for PK assay was followed as described by *Simcox et al., 1977.* [13]

Time (min)	Field		15^0C		25^0C	
0	A1	A2	A3	A4	A5	A6
0.5	0.0248	0.0382	0.1109	0.3393	0.0222	0.0232
1	0.0237	0.0380	0.1093	0.3387	0.0194	0.0214
2	0.0222	0.0376	0.1057	0.3379	0.0132	0.0152
3	0.0219	0.0364	0.1046	0.3356	0.0103	0.0132
4	0.0212	0.0356	0.1008	0.3317	0.0077	0.0107
5	0.0203	0.0334	0.0961	0.3274	0.0043	0.0042

Table 16: *Absorbance readings for Field, 15^0C and 25^0C samples at different time intervals*

Experiment2:

Time (min)	Field		15^0C		25^0C	
0	A1	A2	A3	A4	A5	A6
0.5	0.0248	0.0382	0.0047	0.0043	0.0793	0.0579
1	0.0246	0.0376	0.0045	0.0039	0.0783	0.0568
2	0.0242	0.0375	0.0042	0.0036	0.0775	0.0560
3	0.0239	0.0374	0.0036	0.0032	0.0771	0.0549
4	0.0238	0.0373	0.0035	0.0030	0.0765	0.0546
5	0.0237	0.0372	0.0030	0.0028	0.0764	0.0543

Table 17: *Absorbance readings for Field, 15^0C and 25^0C samples at different time intervals*

Experiment3

Time (min)	Field		15^{0}C		25^{0}C	
0	A1	A2	A3	A4	A5	A6
0.5	0.1786	0.1827	0.1053	0.1535	0.2078	0.1731
1	0.1782	0.1824	0.1041	0.1532	0.2069	0.1719
2	0.1776	0.1821	0.1029	0.1518	0.2066	0.1716
3	0.1775	0.1820	0.1025	0.1512	0.2056	0.1708
4	0.1774	0.1815	0.1023	0.1507	0.2049	0.1704
5	0.1772	0.1813	0.1022	0.1505	0.2044	0.1699

Table 18: *Absorbance readings for Field, 15^{0}C and 25^{0}C samples at different time intervals*

Each experiment was performed in duplicates

No. of times the experiment was repeated = 3

Calculation of $\Delta A_{340/min}$

From the absorbance readings, change in absorbance Δ(A) was calculated as explained above.

	Field(duplicates)		15(duplicates)		25(duplicates)	
ΔA 1	0.0011	0.0002	0.0016	0.0006	0.0028	0.0018
ΔA 2	0.0015	0.0004	0.0036	0.0008	0.0062	0.0062
ΔA 3	0.0003	0.0012	0.0011	0.0023	0.0029	0.0020
ΔA 4	0.0007	0.0008	0.0038	0.0039	0.0026	0.0025
ΔA 5	0.0009	0.0022	0.0047	0.0043	0.0034	0.0065

Table 19: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time interval.*

The same was repeated for Experiment 2 and 3:

	Field(duplicates)		**15(duplicates)**		**25(duplicates)**	
ΔA 1	0.0002	0.0006	0.002	0.004	0.0010	0.0011
ΔA 2	0.0004	0.0001	0.003	0.003	0.0008	0.0008
ΔA 3	0.0003	0.0001	0.006	0.004	0.0004	0.0011
ΔA 4	0.0001	0.0001	0.001	0.002	0.0006	0.0003
ΔA 5	0.0001	0.0001	0.005	0.002	0.0001	0.0003

Table 20: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time intervals*

	Field(duplicates)		15(duplicates)		25(duplicates)	
ΔA 1	0.0004	0.0003	0.0012	0.0003	0.0009	0.0012
ΔA 2	0.0006	0.0003	0.0012	0.0014	0.0003	0.0003
ΔA 3	0.0000	0.0001	0.0004	0.0006	0.0010	0.0008
ΔA 4	0.0001	0.0005	0.0002	0.0005	0.0007	0.0004
ΔA 5	0.0001	0.0002	0.0001	0.0002	0.0005	0.0005

Table 21: *Calculation of change in absorbance (ΔA) for different P.kurroa samples between different time intervals*

PK activity calculation

The enzyme activity was calculated as:

Activity (U/ml)= $\Delta A_{340/min} / \varepsilon_{NADH,340}$

U/ml = units per ml (Units are µmoles/min/ml)

$\Delta A_{340/min}$ = change in absorbance at 340 nm

$\varepsilon_{NADH,340}$ = molar absorption coefficient/ molar extinction coefficient of NADH at 340 nm

$\varepsilon_{NADH,340} = 6.22 * 10^3\ M^{-1}\ cm^{-1}$

$= \Delta A_{340/min}\ (min^{-1}) / (6.22 * 10^3\ M^{-1}\ cm^{-1})$

$= \Delta A340/min\ (min^{-1}) / (6.22 * 10^3\ moles^{-1}\ Litre\ cm^{-1})$

$= \Delta A_{340/min}\ (min^{-1}) / (6.22 * 10^3 * 10^3\ moles^{-1}\ ml\ cm^{-1})$

$= (\Delta A_{340/min} / 6.22) * 10^{-6}\ moles\ ml^{-1}\ min^{-1}$

$= (\Delta A_{340/min} / 6.22)\ \mu moles/min/ml$

$= (\Delta A_{340/min} / 6.22)\ U/ml$

Calculation of specific activity:

Specific activity (U/mg) = Activity (U/mg)/Protein content (mg/ml)

U/mg= units per mg (Units are µmoles/min/mg)

Therefore, specific activity = $(\Delta A_{340/min}) * (1/ \varepsilon_{NADH,340}) * (1/$ Protein content)

Field(U/mg)	15°C(U/mg)	25°C(U/mg)
0.00020	0.00080	0.00062
0.00023	0.00085	0.00063
0.00028	0.00086	0.00067

Table 22: *PK specific activity (U/mg) in Field, 15°C, 25°C samples*

	Field(U/mg)	15°C(U/mg)	25°C(U/mg)
Mean	0.00024±0.00004	0.00084±0.000032	0.00064±0.000026

Table 23: *Average PK specific activity of the three samples*

Analysis:

The PK specific activity of the three *P.kurroa* plant samples follows the order 15°C > 25 °C> field.

PK specific activity was highest in the 15 °C plant.

CHAPTER 5

DISCUSSION

There are various factors which influence the accumulation of secondary metabolites (mainly Picroside I and II) in P.*kurroa*. Effect of altitude on the picroside content of P.*kurroa* in the north western Himalayas has been studied and it has been concluded that picroside accumulation depends on altitude. [14] Additionally, it has been reported that temperature regulate the expression of genes in terpene biosynthesis and also the picrosides levels in *P. kurroa*. Expression of the two regulatory genes of terpenoid metabolism, 3-hydroxy-3-methylglutaryl coenzyme A reductase (pkhmgr) and 1-deoxy-D-xylulose-5-phosphate synthase (pkdxs) was upregulated at 15 ^{0}C as compared to at 25^{0}C. Picrosides content exhibited the trend similar to gene expression. [15]

These studies show that altitude and temperature are the main factors influencing terpenoid metabolism in of *P. kurroa.* [14, 15] Temperature of 15^{0}C favored picrosides accumulation as compared to 25^{0}C. [15]

It was observed in the present study that the specific activities of Phosphofructokinase as well as Pyruvate kinase are highest in 15^{0}C sample.

It can be seen that there is variation in the activity of glycolytic regulatory enzymes and picroside content as the temperature is varied.

This suggests that there may be correlation between the activities of Glycolytic regulatory enzymes and Picroside accumulation in P.*kurroa*.

CHAPTER 6

CONCLUSION

It has been seen in the present study that the 15^0C *Picrorhiza kurroa* plant which has the highest picroside content also has the highest specific activities of PK and PFK(the Glycolytic regulatory enzymes). With alterations in the growth temperatures of *P. kurroa*, the specific activity of PK and PFK change. It may be suggested that these growth temperatures influence the final Picroside content in the plant by affecting the activities of various Glycolytic enzymes. Furthermore, it can be concluded from this preliminary study that alterations in temperature affect not just the secondary metabolite biosynthesis but also the activity of Glycolytic regulatory enzymes in *P. kurroa.*

REFERENCES

Booklet:

[1] A.R. Teal and P.E.O. Wymer, Enzymes and their Role in Biotechnology, Biochemical Society, London.

World Wide Web:

[2] http://www.chem.qmul.ac.uk/iubmb/enzyme/rules.html

Journal article:

[3] C. Winkler, B. Delvos, W. Martin and K. Henze, "Purification, microsequencing and cloning of spinach ATP-dependent phosphofructokinase link sequence and function for the plant enzyme', FEBS Journal, vol. 274, pp. 429–438, 2007.

World Wide Web:

[4] http://proteopedia.org/wiki/index.php/Phosphofructokinase (PFK).

Journal article:

[5] S.G. Gottlob-McHugh, R.S. Sangwan, S.D. Blakeley, G.C. Vanlerberghe, K. Ko, D.H. Turpin, W.C. Plaxton, B.L. Miki and D.T. Dennis, "Normal Growth of Transgenic Tobacco Plants in the Absence of Cytosolic Pyruvate Kinase", Plant Physiol. , vol. 100, pp. 820-825, June 1992.

World Wide Web:

[6] http://www.proteopedia.org/wiki/index.php/Pyruvate_Kinase

[7] http://www.herbal-supplement-resource.com/picrorhiza-kurroa-root.html

Journal article:

[8] R. Kumar , P. Bhandari, B. Singh and P.S. Ahuja, "Evaluation of Picrorhiza kurrooa accessions for growth and quality in north western Himalayas" , Journal of Medicinal Plants Research ,vol. 6(13), pp. 2660-2665, April 2012.

Booklet:

[9] Sayeed Ahmad, "PHARMACOGNOSY: Introduction of Plant Constituents and their Tests", Jamia Hamdard, India, July 2007.

Journal article:

[10] H. Sood and R.S. Chauhan, "High frequency callus induction and plantlet regeneration from different explants of *Picrorhiza kurroa* - a medicinal herb of Himalayas" , African Journal of Biotechnology ,vol. 8 (9), pp. 1965-1972, May 2009.

[11] H. Sood and R.S. Chauhan, "Biosynthesis and accumulation of a medicinal compound, Picroside-I, in cultures of *Picrorhiza kurroa* Royle ex Benth", Plant Cell Tiss Organ Culture, vol. 100, pp. 113–117, 2010.

[12] W.C. Plaxton, "The organization and regulation of Plant Glycolysis", Annu. Rev. Plant Physiol. Plant Mol. Biol., vol. 47, pp. 185–214, 1996.

[13] P.D.Simcox, E.E. Reid, D.T. Canvin and D.T. Dennis, "Enzymes of the Glycolytic and Pentose Phosphate Pathways in Proplastids from the Developing Endosperm of Ricinus communis L.", Plant Physiol., vol. 59, pp.1128-1132, 1977.

[14] M. Katoch , I.S. Fazli , K.A. Suri , A. Ahuja and G.N. Qazi, "Effect of altitude on picroside content in core collections of Picrorhiza kurrooa from the north western Himalayas" , J Nat Med, vol. 65(3-4), pp. 578-82, July 2011.

[15] T. Kawoosa , H. Singh, A. Kumar, S.K. Sharma, K. Devi, S. Dutt, S.K. Vats, M. Sharma, P.S. Ahuja , S. Kumar, "Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpene picroside accumulation in *Picrorhiza kurrooa*", Funct Integr Genomics, vol. 10(3), pp.393-404, August 2010.

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