CHAPTER VIII: BIOLOGICAL ACTIVITY OF LEAF EXTRACT OF *FAGOPYRUM DIBOTRYS* (D DON) HARA.

Introduction.

The realization of the existence of active principles in plants as decided by the mankind was a great step towards understanding the existence of phytomedicine .The method of isolation of secondary products presumed the different shape in the greater stride taken in the development of herbals and opened a new vista of phytotherapy. In many parts of India, the plants are used by traditional medical practitioners for the treatment of various diseases (Singh et al . 1984 Asolkar et al 1992 ;Pradhan and Basu . 1998) . Though medicinal use of *Fagopyrum dibotrys* (D.Don) Hara was reported earlier in the literatures by Kiritkar and Basu (1983); Chopra et al, (1956); Rastogi and Malhotra, (1995) but during survey in Darjeeling and Sikkim hills many unknown ethnobotanical information's have been collected as represented in Chapter VII. Recently interest in "folk " or traditional medicine has been revived all over the world, since future trend is more towards utilisation of herbal medicine instead of allopathic medicine because of the fact that certain diseases are not cured by allopathic medicine. Besides, they have side effect as compared to herbal medicine. Uptodate, large number of natural products have been isolated and characterised but the knowledge in connection with understanding of bioactivity of natural products is meager.

Here in this part of work an attempt has been made to explore the bioactivity of plant extract so that the knowledge gained out of it will be of much help in connection with scientific evaluation of *Fagopyrum dibotrys* (D Don) Hara.

Section A: Immunological observation in albino rat subjected to water extract of leaf of Fagopyrum dibotrys (D.Don) Hara

Material:

Leaf of *Fagopyrum dibotrys* (D. Don) Hara of both Darjeeling and Gangtok varieties and male rats of twelve weeks of age.

Methods

i) Preparation for intravenous injection to albino rat.

The leaves of *Fagopyrum dibotrys* (D.Don) Hara were extracted with methanol. which later made soluble into water. This water-soluble fraction was made in the different concentrations. The different concentrations of water soluble part of methanol extract of *Fagopyrum dibotrys* (D.Don) Hara leaves were used for the immunological study on the albino rats. It was extracted in sterilized distilled water and was passed through millipore membrane filter with 0.45 m porosity before intravenous injection.

ii) Preparation of blood sample for lymphocytes count

Peripheral blood was collected in 3.13% sodium citrate solution from each of the experimented trials. . Erythrocytes in peripheral blood were lysed by exposure to tris- buffer 0.83% ammonium chloride solution pH7.2 for 10 minutes and finally washed thoroughly with phosphate buffer saline pH 7.0-7.2. and spun down at 3000 rpm for 15 minutes for separation of lymphocytes from R.B.C s . debris etc .and measurement of the blastogenic transformation of lymphocytes as percentage of blast cells was calculated after counting the viable medium plus large lymphocytes out of every 100 of total viable lymphocytes on haemocytometer in presence of trypan blue. Cells with diameters greater than approximately 7 um were scored as medium sized and

cell with diameter greater than 10-11 um were scored as large (Chakravarty and Clark, 1977). Normal blast cells were 3 to 6 %.

Section B : Bilirubin content in the blood samples of tribal people taking water decoction of leaf of *Fagopyrum dibotrys* (D.Don) Hara

Material

Leaves of *Fagopyrum dibotrys* (D.Don) Hara

Methods:

a) Blood sample collection :

The blood was collected from the jaundice patient at different interval of treatments upto 13 days along with the treatment of tribal medicine by the application of leaf extract of *Fagopyrum dibotrys* (D.Don) Hara

b) Estimation of bilirubin.

Estimation was made following Malloy and Evelyn method (1937).

c) Detection of bilirubin in urine samples collected from tribal people.

i) Urine samples collection.

The urine samples were collected from the hyperbilirubinemia patient.

ii) Categorization of hyperbilirubinemia.

For the detection of bilirubin and to categorize the type of hyperbilirubinemia the methodology of Malloy and Evelyn method (1937) was used.

Section C: Antifungal activity of the rhizome extract of *Fagopyrum dibotrys* (D Don) Hara.

Material.

Rhizomes of *Fagopyrum dibotrys* (D.Don) Hara.

Methods.

a) Plating of the soil collected from the rhozophere of *Fagopyrum dibotrys* (D Don) Hara.

The soil washing was performed using perspex boxes fitted with 3 stainless sieves of graded size .A soil sample was serially washed under aseptic conditions by passage of sterile compressed air through the boxes. The resulting agitation gradually broke up aggregates and released spores held by them. On completion of washing, discrete soil particles were distributed on the sieves according to their plated, to flourish the fungal hyphae in potato dextrose agar.

***b)* Effect of the rhizome water extract on the growth of *Aspergillus niger*.**

Using 24 hours old cultured slants of the mould (*Aspergillus niger*) as indicator organisms isolated from soil. The small circular cavities were prepared with the help of borer the size of which were 3mm in diameter. The small cavities were filled with the rhizome water extract. The rhizome water extract was prepared by boiling 1gm of rhizome in 150 ml volume of water till the volume reached upto 100 ml .The slants were kept for the observation upto 2-5 days after incubating at 30 ^{0}C.

Results and Discussion.

Immunostimulation constitutes an attractive alternative to conventional chemotherapy and prophylaxis when the hosts defence mechanisms have to be activated under conditions of impaired immune responsiveness. (Drews. 1980; Wagner. 1982). Taking consideration of these view. the injection of water extracts of *Fagopyrum dibotrys* (D. Don) Hara leaves of the Darjeeling and Gangtok varieties were performed separately and show similar immunological activity as represented in the Graph 10. Injection was made on the albino rats at different concentrations of water extract as 50 μg. 100 μg. 200 μg. 500 μg and 1000 μg.

The increase of blast cells was measured from the second day of injection to fourth day. After the twenty four hour of study 50 μg. 100 μg 200 μg and 500 μg of water extracts had 17%. 20% 5% and 40% increase of the blast cells. respectively whereas the 1000 μg had 10% of the increase in blasts cells. After the forty eight hours of the observation the treatment of 50 μg. 100 μg 200 μg. 500 μg and 1000 μg had 28%, 22 %. 26%, 63% and 82 % of increase in blast cells. respectively. On the third day. the same order of the concentrations i.e 50 μg. 100μg. 200μg. 500μg and 1000 μg .The increase in percentage of blasts cells were 31%. 28%. 50%. 50%and 92%respectively and in the fourth day. it were 39%. 30%. 58%. 40% and 80%. respectively (Fig 10) .In application of 50μg of Con A to the animal could initially boost the level of blastogenesis (Waterfield et al. 1975 and Waterfield andWaterfield. 1976) but these cells became physiologically exhausted and died (Choudhuri and Chakravarty.1981).Similarly . it was found that the water soluble part of methanol extract of 1000 μg induced the percentage of blasts cells after second day of treatment but 200 & 500 μg of water soluble part of methanol extract had maximum 63 % and 59% increase in the blasts cells percentage which would reach after second day and fourth day . respectively .Water extract of 200 μg therefore be considered as

optimum to show maximum activation of lymphocyte to blast cells and which could be suitable for the immunological development .

Methanol extract of leaf of *Fagopyrum dibotrys* (D Don) Hara was worked out earlier to contain and found to contain rutin (Imai and Furuya, 1951). quercitin (Yamato and Koyama, 1962). cyanidin 3 glucoside (Basu and Pradhan, 2000) cyanidin 3, 5 diglucoside (Basu. 1997) and β sitosterol (Basu. 1997). The bioactivities of these isolated natural products have been worked out after isolating the same products from other plants. Their reported bioactivities from other plants are anti –cancer (Yun Peng et al.. 1989). anti mutagenic (Dauer et al.. 1998. Lee et al 1998.) immunomodulating activity (Beuth et al., 1995). aldose reductase (Underhill. 1957). to reduce the haemorhages in the eye. reducing the risk of stroke (Griffith. 1955). to reduce the anaemia and leucocytes (Rokers and Marti (1951) and inhibitory role of leukaemic H L 60 cell growth (Hirano et al. 1995). The phenolic acids such as 2.3 dihydroxybenzoic acid was observed to stimulate the phagocytotic activity of polymorphonuclear granucytes .The peptide bound water soluble heteroglycans possessed the anti-tumour activity ,activation of polyclonal B, lymphocytes and enhancement of antibody .(Kumazawa.1982).Quercitin inhibits the D.N.A synthesis of human leucomia cells.(Uddin and Choudhury.1995). Similarly . many workers viz. Fuhrman (1955). Crimson (1948),Fukuda (1932) Sokoray and Czimmer (1938), Armentuno,(1936) Sood et al (1982) . Benko (1970),Takahasi (1998) Bland (1984) .Wagenbreth .et al (1996) , Meyer . et al.. (1998a) studied the bioactivities of the phenolic compounds.

However, the result of T lymphocyte activation by water extract of the plants led to consider *Fagopyrum dibotrys* (D.Don) Hara as the immunostimulatory drugs. Whistler et al., (1976) and Haba et al.. (1976) had reported that the anti-tumor activity performed by the activation of the T-lymphocyte .On the contrary, it might be suggested that the activation of T

lymphocytes considered had some role on the anti-tumour as found by Yoa et al (1989) who isolated epicatechin 3 galloyl – (-) epicatechin. procyanidin and 3. 3′ digalloyl procyanidin as antitumour constituents of *Fagopyrum dibotrys* (D Don) Hara. It was similarly reported by Yung-Peng et al (1989) from this plant. Immune response to malignant tumour has been the subject of study of immunologists. which received its major impetus from 1960s. Non-specific approaches were directed mostly towards stimulating the reticulo-endothelial system of hosts with the help of agents of plant origin (Fletcher et al 1980). The function of graft rejection. including tumour grafts has been ascribed to the T lymphocytes (Daynes et al. 1979; Herberman et al 1980: Keder and Weiss. 1981: Prowse et al. 1983). As the stimulation of these cells promises more of a specific approach. the activation of lymphocytes in the blood caused by plant product is considered to be related to a site to antitumour activities.

No immunological work utilising *Fagopyrum dibotrys* (D.Don) Hara has been done earlier except Liu et al (1981) who. reported the increase of phagocytic activity in mice and rats by 5. 7.3`. 4` tetrahydroflavan 3-ol dipolymer.

On the survey in the different interior hills of Sikkim and Darjeeling Himalayas. it was found that various plants had been using against different diseases. the information of which was unknown to the world. Moreover, the concept of Lepcha (Medicinemen) treatments never been considered as scientific approach but according to some authors the Lepcha are known with the use of medicinal herbs (Hooker. 1856. Biswas. 1956. Foning. 1987). During survey, there was some contact with Lepcha medicineman and it was observed that they used the plant for the treatment of jaundice. In order to verify the role of the plant on jaundice, the bilirubin content was estimated following Malloy and Evelyn (1937) method. The normal bilirubin content in the human being is establish to become 0.3– 1.0 mg/dL. Acrossing this range of bilirubin content in the blood would be resulted with the jaundice

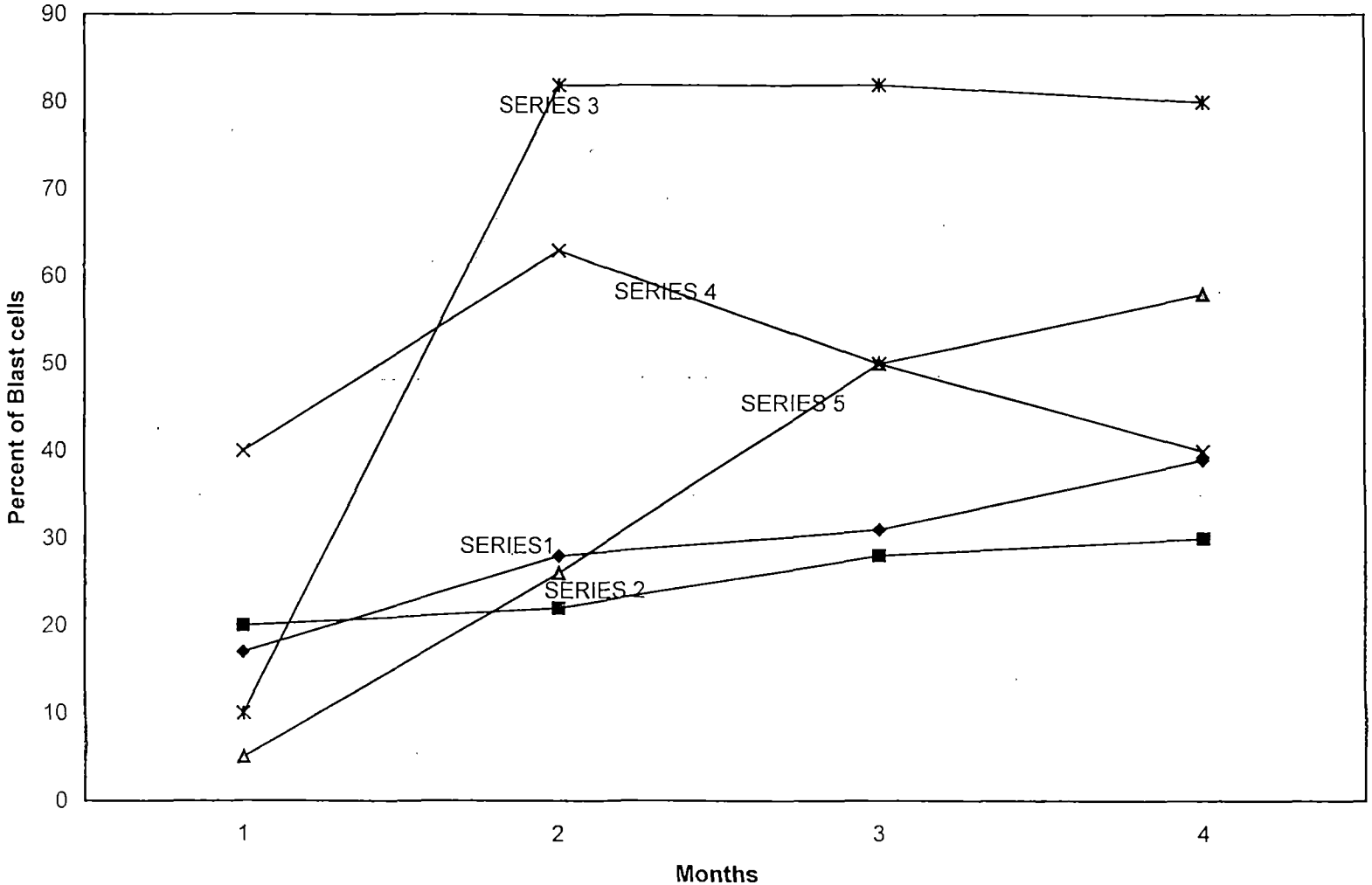
disease (Kalpan and Isselbacher, 1998). The information on the bilurubin in the blood plasma through the estimation seems that the plant has potentiality to cure the jaundice as shown in the Graph 11 .The blood samples were collected from ten patients who used to take 10 ml extract prepared from dried leaf (5.8 gm) of *Fagopyrum dibotrys* (D Don) Hara twice a day after their meals and was also collected the blood from the normal healthy without jaundice person. Initially upto 5 days . no mark change in bilirubin content was observed as compared to control but it came near to the normal after the seventh days of treatment with bilurubin 1.3 mg /dL . On the ninth day. the content of bilirubin was very much lowered to 0.75 mg/dL within the normal range .The tribal medicineman generally continued their treatment for 13 days .On the 13^{th} day the patient was observed to contain the bilirubin content only 0.45 mg/ dL which was well within the normal range. Similar observation was noted after the results gathered from the treatment of Gangtok variety. This seem that all the bioactive products of both Darjeeling and Gangtok varieties may be acting on the enzymatic system or any other physiological system of the body in the similar manner. Upto date no information in connection with the cure of jaundice by the utilization of the extract of leaf of *Fagopyrum dibotrys* (D.Don) Hara is available. Thus this may be treated as a new report on the remedial activity of the plant .In fact . the accumulation of bilurubin in the bloodstream causes the symptom of the jaundice but understanding the nature of jaundice is a problem. Following the simple method of chemical determination of bilurubin . by which jaundice can be identified noting either presence or absence of bilirubin in urine of the collected sample (Kaplan and Isselbacher. 1998).No bilirubin was detected from the urine sample confirmed that the jaundice is of unconjugated hyperbilubinemia .

It is well established that the circulating bilurubin approximately 80 %is derived from red blood cells and approximately 15 to 20 percent of circulating bilurubin is derived from other sources. including (1) ineffective erythropoiesis resulting from destruction of maturing erythroid cells in the

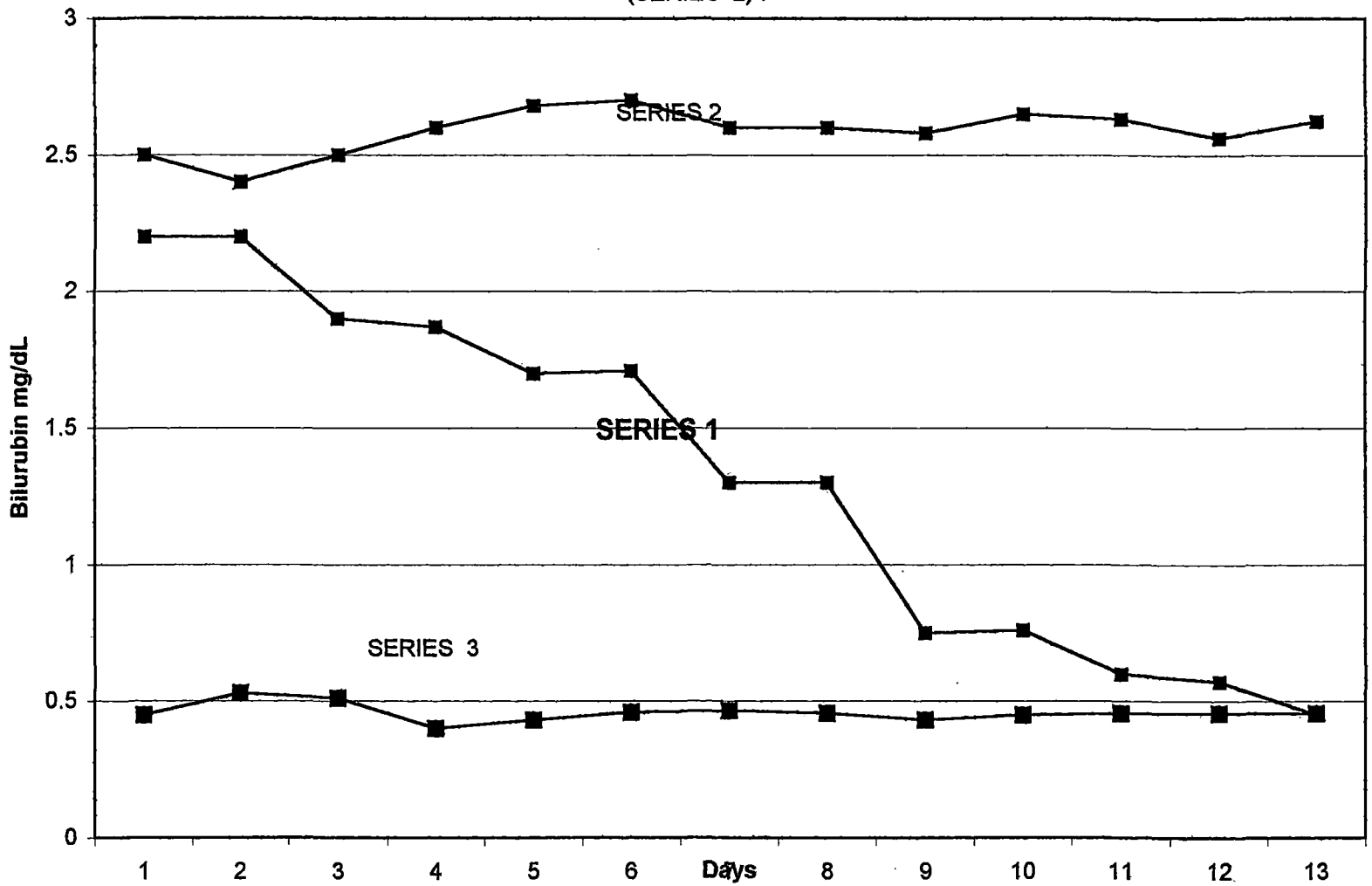
bone marrow; and (2) the metabolism of other heme-containing proteins, most notably hepatic cytochromes, muscle myoglobin, and widely distributed heme-containg enzymes. A number of works on the bilurubin metabolism has been found with the information about the central role of liver in the metabolism of the bile pigments (Choudhury et al. 1993; Depagter et al. 1976; LaMont and Isselbacher, 1992) .The role has generally been divided into three distinct phases: (1) hepatic uptake (2) Conjugation and (3) excretion into bile. The uptake and subsequent hepatocyte storage of bilurubin involve binding of bilurubin to cytoplasmic anion –binding proteins , especially ligandin (glutathione –S –transferaseB), that prevents efflux of bilurubin back into the plasma . The presence of glucuronosyl transferase in the hepatocyte for the metabolic process of bilurubin has vital role in within the endoplasmic reticulum. (Choudury, 1995; Kaplan and Isselbacher, 1998) .The impaired of these enzyme could led unconjugated bilurubinemia but not conjugated bilurubinemia (Kaplan and Isselbacher, 1998; Isselbacher, 1998; Managhang et al. 1996) on the otherhand interference with the billary excretion of conjugated bilurubin by hepatocytes leads result only conjugated bilurubinemia (Kaplan and Isselbacher, 1998).

While in the experimental study of Kiyasheva (1974) with daily oral administration of rutin (100 & 200 mg /kg) and quercetin (100 –200 mg/kg) for the 30 days found the increase of the biliary secretion, excretion of bile acids and cholesterol in bile. In other experiment, Siess et al. (1988) found that flavone increased the liver weight with hepatic cytochrome P450 in rats following a 14 day treatment with 0.25 % flavone in feed and also reported the flavone activity which also increased the hepatic monoxygenase activities and UDP glucuronyltransferase activity, by both quercetin and flavone .It seemed that the Rutin, quercetin and flavone play some roles in the bile metabolism.

GRAPH 10 : ACTIVATION PERCENTAGE OF LYMPHOCYTES BY WATER SOLUBLE EXTRACT OF *FAGOPYRUM DIBOTRYS* (D.DON) HARA [SERIES 1 -50 ug ;SERIES 2 100 ug ; SERIES 3 200ug ; SERIES 4 500 Ug and series 5 1000 ug].



GARPH 11 : ESTIMATION OF THE BLOOD BILIRUBIN CONTENTS IN JAUNDICE PATIENTS (SERIES 1) AFTER TREATMENT OF TRIBAL MEDINE PREPARED BY WATER DECOCTION OF *FAGOPYRUM DIBOTRYS* (D.DON) HARA LEAVES,TAKING STANDRADS OF BOTH NORMAL (SERIES 3) AND UNTREATED JAUNDICE PATIENT
(SERIES 2) .



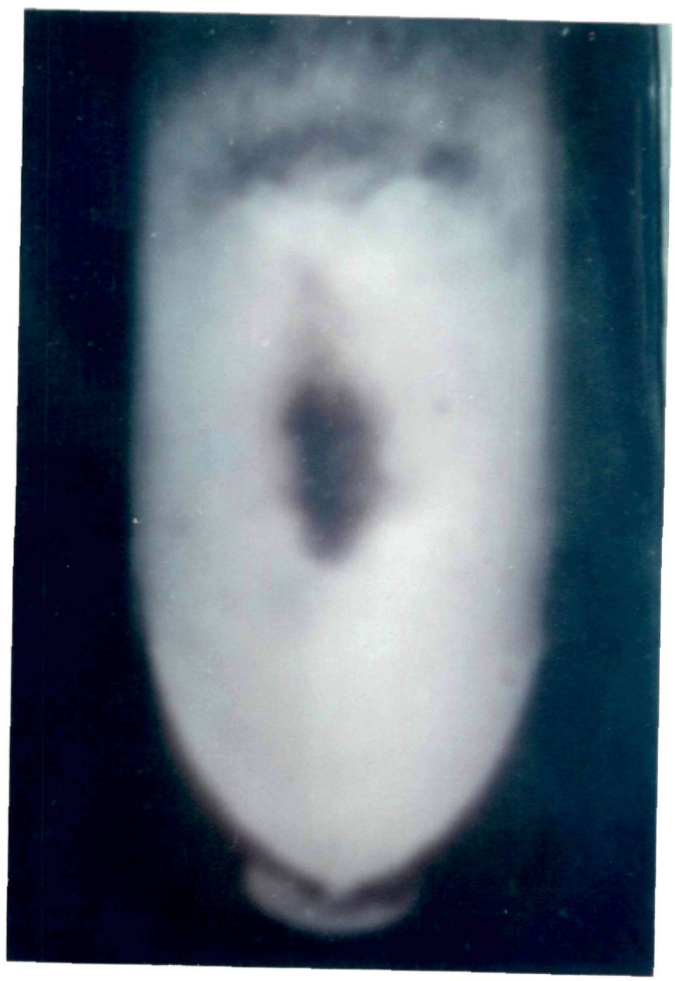


Fig 34 .Showing the mat of *Aspergillus niger* with black spot after application of *Fagopyrum dibotrys* (D.Don) Hara rhizome water extract .

The leaves of *Fagopyrum dibotrys* (D Don) Hara leaves have been reported to contain rutin (Furuya, 1954; Basu, 1997), cyanidin 3 glucoside, cyanidin 3, 5 diglucoside (Basu, 1997), β sitosterol (Basu, 1997) and quercetin (Yamato and Koyama, 1962) It is expected that the flavonoid present in the plant may have certain role to decrease the bilurubin content of the blood plasma by acting on the enzymes glucunosyl transferase.

An antifungal activity was also seen after the application of water extract of rhizome of *Fagopyrum dibotrys* (D Don) Hara rhizome on the culture of *Aspergillus Niger* . *Aspergillus niger* was separated from the soil of plant habitats and grown in the potato dextrose agar medium. The bed of *Aspergillus niger* was turned into black after 24 hours when the rhizome water extract was applied into the small cavity (3mm) of culture tubes (Fig34). However, the potentiality of antimicrobial activities was found to be decreased with the dilution of the extract. It is supported by the works of Heimann et al, (1953) and Swain (1977) who found antifungal activities of procyanidin. The presence of procyanidin was reported by Liang and Xiao, 1990 in the rhizome that might be a reason for such activity.

The tribal claim of ethnomedicine to *Fagopyrum dibotrys* (D. Don) Hara of both Darjeeling and Gangtok varieties were tested and found related with the number of biactivities such as, immunological, pharmacognological and antimicrobial. Though it has been known that is a variation in chemical composition between the Darjeeling and Gangtok varieties of *Fagopyrum dibotrys* (D. Don) Hara but no difference was found as for their results obtained from bioactivities tests .These is an attempt to establish whether the plant has got any medicinal values or not and to find out the real importance in terms of commercial point of view .

Summary

The biological activity of water soluble part of leaf of *Fagopyrum dibotrys* (D.Don) Hara was studied to understand its efficacy from immunological point of view, which acceralate the activation of lymphocytes to blast cells.

The Lepcha medicinemen were also consulted and found that the dried leaf of *Fagopyrum dibotrys* (D .Don) Hara were being used by them for the treatment of jaundice. The experiment was carried with the help of medicinemen and the blood samples were collected from the jaundice patient treated or untreated with the leaf extract .The result after the determination of the bilirubin content in the blood confirmed that the jaundice could be cured with leaf of *Fagopyrum dibotrys* (D .Don) Hara and it was effective against unconjugated hyperbilirubemia.

Antifungal activity of rhizome extract was also observed.