Chapter-4

Results and Discussion

4.1 Plant Material

The morpho-anatomical features of *Hodgsonia heteroclita* fruit is as given in the figure- 4.1. The fruit size is about 12cm plate. Longitudinal section shows whitish flashy fruit pulp. Transverse section of fruit shows six large seed with reduced accompanied seeds for which it seems the plano-convex shape. There are whitish oily oval shaped dicotyledons inside the woody hard seed coat.

4.2 Plant Extract Yield

After complete soxhletion using the hydro-methanolic solvent and rota-evaporation, the total amount of drug extract in its crude form was yield. The quantity of crude drug was calculated as follows-

Calculation of drug extract:

Weight of crucible = 46.55gm

Weight of crucible + weight of extract =133.78g

Therefore, weight of the extract

= (Weight of crucible + Weight of extract) –Weight of crucible

=133.78g - 46.55g

= 87.23g

Percentage of yield obtained

= (Weight of extract / Weight of powdered peel) x100

= (87.23/300) x 100

=29.07% yield

Therefore, the percentage yield of methanolic extract was found to be 29.07% (87.23g) weight.

4.3 *In vitro* Studies

4.3.1 Preliminary phytochemical screening of extract: The preliminary phytochemical screening of 70% methanolic drug extract revealed the presence of various phytoconstituents. The appearance of different phytoconstituents of the test samples revealed the presence of various ranges from moderate to high. Among the screened biochemicals flavonoid content was inferred highest as given in the table-4.1.

Plants are the rich sources of different phytochemicals having varied biological activities that can be of valued in both food and medicine. Extracts of different plants may contain different phytochemicals having its unique biological activities (Harborne 1998). The phyto-chemicals are reported to perform

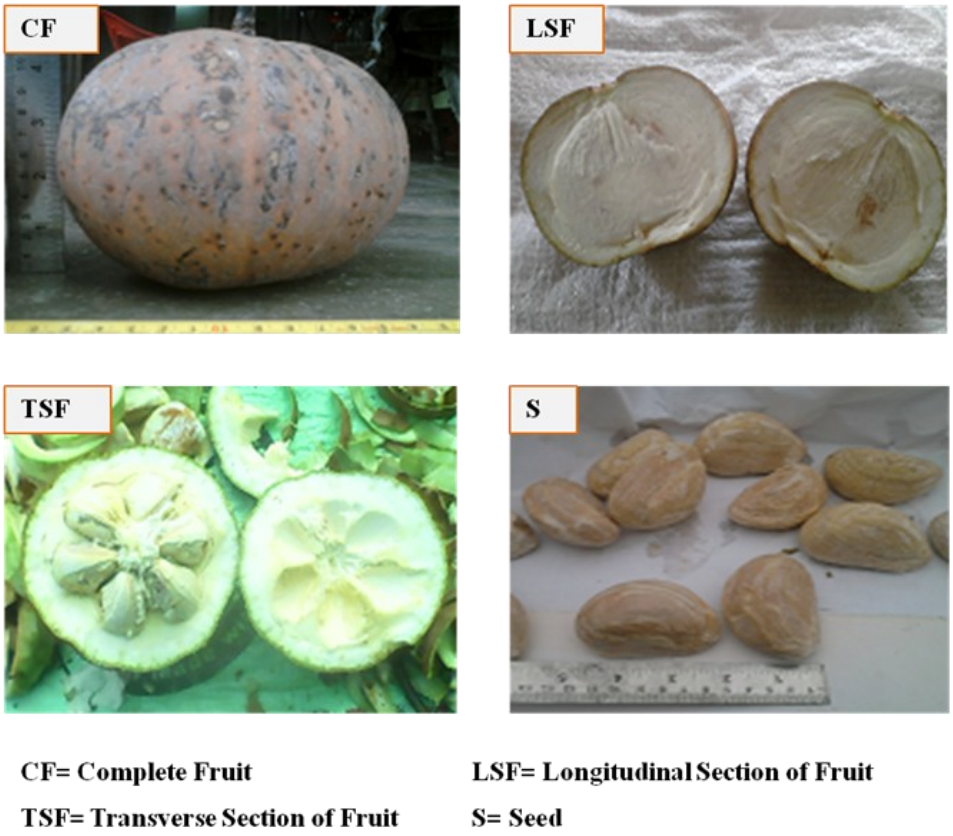


Fig: 4.1- Morpho-anatomical features of *Hodgsonia heteroclita* fruit

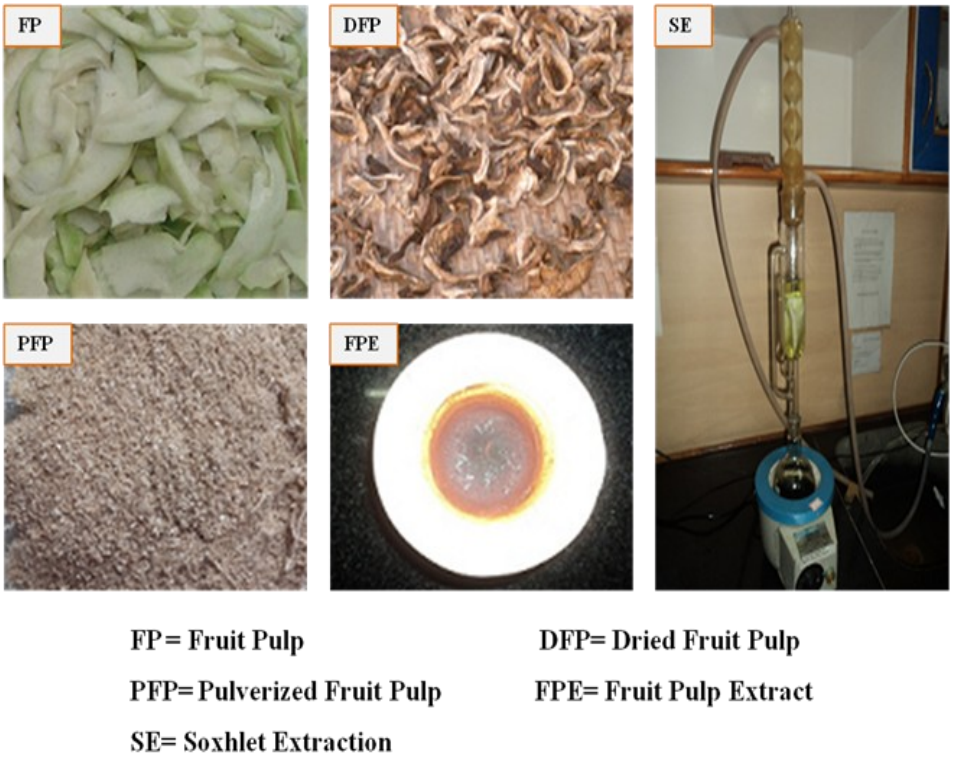


Fig: 4.2- Processed fruit pulp of *Hodgsonia heteroclita*

Table-4.1: Appearance of biochemical constituents of HFP

Sl. No.	Chemical compounds	Results
1.	Saponins	+
2.	Steroids	+
3.	Alkaloids	+
4.	Tannins	+
5.	Carbohydrates	+
6.	Flavonoid	++
7.	Anthraquinone	+
8.	Glycosides	+
9.	Reducing sugars	+

[+=Moderate detected; ++= high detected]

several important functions in different biological and pharmacological actions in the animals when consumed. The biochemical constituents present in fruit and vegetables have received considerable importance because they possess a broad spectrum of chemicals and biological actions (Halliwell 2007). These actions range from cell toxicity to cell protective effects. One of the major functions of these phytochemical is the role of antioxidants (Zhishen et al. 1999). Plant saponins are widely distributed amongst plant that exerts a wide range of pharmacological activities including expectorant, anti-inflammatory, vasoprotective, hypocholesterolemic, immunomodulatory, hypoglycemic, molluscicidal, antifungal, antiparasitic etc. (Sparg et al. 2004). Tannins the water-soluble polyphenols have anticarcinogenic and antimutagenic activity along with antimicrobial properties. It has also been reported to exerts other physiological effects, such as acceleration of blood clotting, reduction of blood pressure, decrease of the serum lipid level, production of liver necrosis and modulate immunoresponses (Chung et al. 1998). Steroids acts as a hormone, it regulates carbohydrate metabolism and has an anti-inflammatory effect in body. Some steroids maintain the blood pressure and regulate the salt

and water balance in the body (de Bodo and Altzuler 1956). Carbohydrate has many major functions within the body, it provides the energy and regulates the blood glucose. Sparing the use of proteins for energy breakdown of fatty acids and preventing ketosis (P Jequier 1994). Glycoside is an important medicinal agent, used in the chronic heart failure, arterial fibrillation supraventricular tachycardia (Ganpisetti et al. 2016). Reducing sugar has the capability of acting as a reducing agent because it has a free aldehyde or a free ketones group. All monosaccharides are reducing sugars along with some disaccharides, oligosaccharides and polysaccharides (Pratt and Cornely 2004). For the fast decades reducing sugars like fucoidans has been extensively studied due to the different biological activities including anticoagulant and antithrombotic, antivirus, antitumor and immunomodulatory, anti-inflammatory, blood lipids reducing, antioxidant and anti complementary properties, activity against hepatopathy, uropathy, renalpathy, gastric protective effects and therapeutic potential in surgery (Li et al. 2008). The alkaloids have been long recognized as an important group of metabolite because of various biological activities like analgesic properties. Flavonoids are one of the largest and widespread groups of secondary metabolite which have been known to have antioxidant, antibacterial, antifungal and antiviral activities. (Podolak et al. 2010). Anthraquinones have been associated with anticancer, laxative and antiarthritic properties (Ebbo et al. 2014).

It is already established that each and every phytochemicals have one or the other biological property. The traditional use of *Hodgsonia* fruit pulp as antibacterial and antidiabetic might be due to the presence of flavonoid and saponin either individually or in conjunction with other compounds.

4.3.2 Determination of Biochemical Constituents: The total phenolic, flavonoid and flavonol contents of 70% methanolic extract of the *Hodgsonia* fruit pulp extract revealed the presence of 15.72±0.01 mg/g GAE phenolic contents, 167.95±0.02 mg/g QE flavonoids and 74.6±0.01 mg/g QE flavonol contents respectively. The crude hydro-methanolic extract of

Hodgsonia fruit pulp revealed the presence of abundant phytocons-tituents. Thus the *H. heteroclita* fruit pulp has a potent source of phenolic antioxidant which may prevent the oxidative free radicals in the living organisms.

4.3.3 Determination of *in vitro* antioxidant potentials: The evaluation of antioxidant potential of *Hodgsonia* fruit pulp revealed the presence of potent source of natural antioxidants. The assessment of antioxidative scavenging activity of 70% methanolic extract with DPPH radical in comparison to standard ascorbic acid showed the enough antioxidative potential. The scavenging activity of ferric reducing power assays of fruit extract in comparison to BHT also revealed the presence of enough potentials of natural antioxidants in *Hodgsonia heteroclita* fruit pulp. The scavenging potentials of DPPH and RPA are represented in the given table-4.2 and the figure-4.3

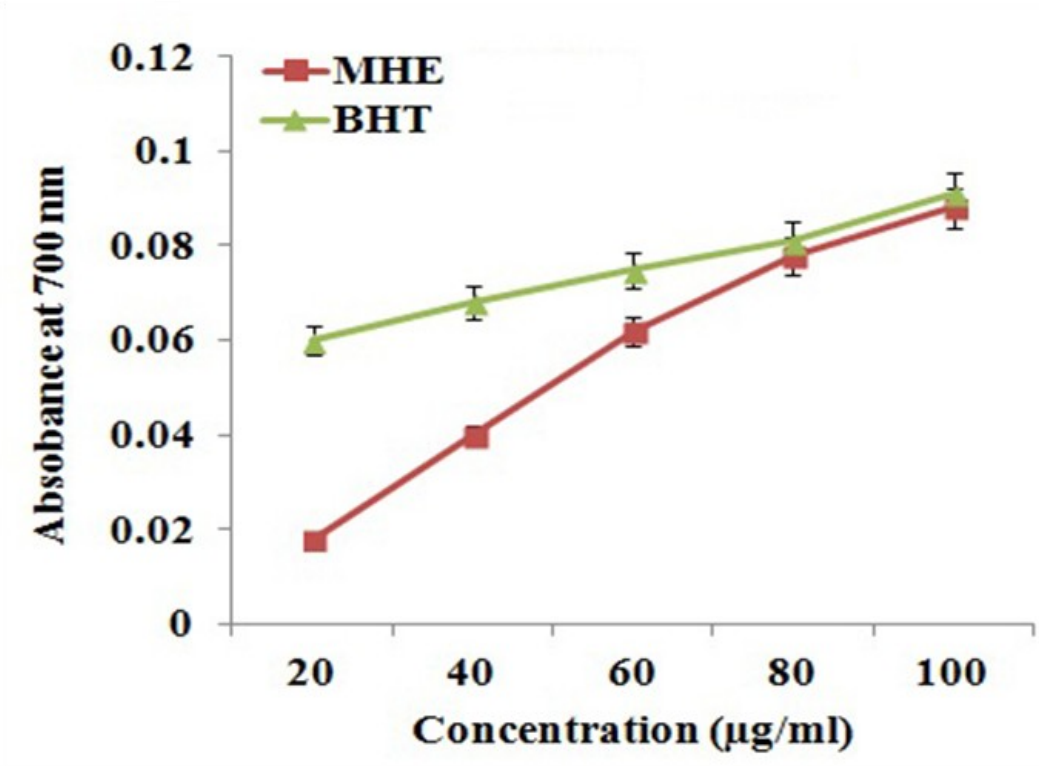
The natural antioxidants have been reported as a potential source for pharmacology (Halliwell 2007). But the availability and suitability to consumptions are not well recognized. So to claim as appropriateness for utilization of orthodox drugs it is necessary to evaluate the efficacy in both *in vitro* and *invivo* systems (Chan and Cheung 2000). The DPPH assay is the reduction of a stable free radical DPPH. The reduction of a stable free radical DPPH with an odd electron gives a maximum absorption at 517nm (purple colour). When antioxidants react with DPPH a stable free radical become paired off in presence of a hydrogen donor and is reduced to the DPPH-H and as the consequence the absorbances decrease from the DPPH (Ionita 2005). The formation of DPPH-H radical results in depolarization with respect to the number of electrons captured. More the depolarization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of new drug (Wagner et al. 1996).

Reducing power assay is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in the antioxidant activity. In this method, antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride. The pale yellow

Table-4.2: DPPH scavenging activity of extract compared with standard

Sl. no.	Conc. in µg/mL	HME	Standard (AA)
1.	20	22.99±1.11	21.12±1.82
2.	40	23.03±2.92	28.98±0.99
3.	80	28.65±0.99	45.98±2.88
4.	120	29.65±2.98	60.16±2.96
5.	160	38.31±1.93	65.90±4.74
6.	200	48.15±3.32	71.13±3.87

[Where, MHE= methanolic *Hodgsonia* extract; AA= ascorbic acid]



[Where, MHE= methanolic *Hodgsonia* extract BHT=Butylated hydroxytoluene]

Fig-4.3: Reducing power assay of extract compared to BHT

reaction mixture turned to the yellowish green. Increase in absorbance of the reaction mixture indicates the reducing power of the samples. In this reaction Fe^{3+} transforms to Fe^{2+} in presence of extract and the reference compound.

4.3.4 GCMS analysis of methanolic extract of *Hodgsonia* fruit pulp: The GCMS is an analytical technique which is a combined capability of physical separation by gas chromatography (GC) with the mass analysis capabilities by mass spectrometry (MS). It is a technique oriented towards the separation, general detection, potential identification and estimation of chemical compounds of particular masses in the presence of other chemicals. A mass chromatogram is a representation of mass spectrometry data as a chromatogram, where the X-axis represents time and the Y-axis represents signal intensity.

The GCMS analysis of methanolic extract of *H. heteroclita* fruit pulp revealed the presence of many bioactive compounds. The GCMS analysis identified 12 bioactive compounds. The identified bioactive phytoconstituents are renown antioxidative as well as glucose lowering compounds. Along with the identified compounds the chromatograms represented many more unidentified bioactive compounds. The identified phytoconstituents are arranged with their common name, chemical formula, IUPAC name, chemical structure, retention time (RT), height occupied, area occupied, concentration (peak area %) as given in the table 4.3a and 4.3b. The chromatograms of the identified compounds are shown in the figure-4.4a, 4.4b, 4.4c, 4.4d, 4.4e, 4.4f, 4.4g and 4.4h. The ascending order of the area occupied by identified compounds in chromatogram are as follows-

1. Protocatechuic acid (871.80) >
2. Salicylic acid (213.23)>
3. o-Coumaric acid (62.60) >
4. Syringic acid (36.79)
5. p-Hydroxy benzoic acid (36.69)>
6. Vanillic acid (25.86)>
7. 2,4-Dihydroxybenzoic acid (25.42)>
8. Ferulic acid (23.34) >
9. p-Coumaric acid (22.52)>
10. Gallic acid (20.06)>
11. Caffeic acid (15.18)>
12. Gentisic acid(11.93)

Table-4.3a:Identified compounds of GCMS analysis of HFP

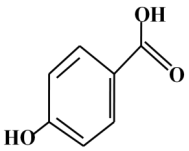
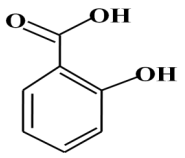
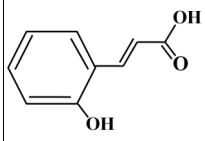
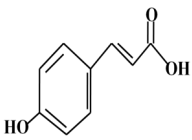
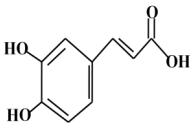
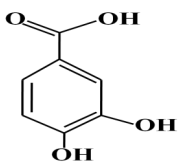
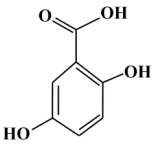
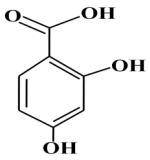
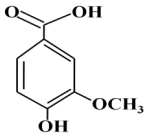
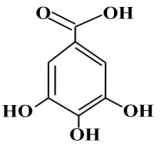
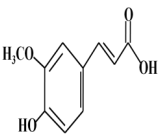
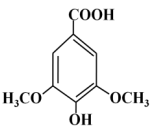
Sl. No	IUPAC Name Chemical Formula Common Name	Chemical Structure	RT (min)	Height	Area	Peak Area %
1	4-Hydroxybenzoic acid HOC_6H_4COOH **p-Hydroxy benzoic acid**		**3.41**	**217**	**36.69**	**14.68**
2	2-Hydroxybenzoic acid $C_6H_4(OH)COOH$ **Salicylic acid**		**6.77**	**530**	**213.23**	**85.32**
3	(2-hydroxyphenyl) prop-2-enoic acid $C_9H_8O_3$ **o-Coumaric acid**		**5.22**	**732**	**62.60**	**73.55**
4	(E)-3-(4-hydroxyphenyl)-2-propenoic acid $C_9H_8O_3$ **p-Coumaric acid**		**5.45**	**441**	**22.52**	**26.45**
5	3-(3,4-Dihydroxyphenyl) -2-propenoic acid $C_9H_8O_4$ **Caffeic acid**		**4.70**	**393**	**15.18**	**100.00**
6	3,4-Dihydroxybenzoic acid $C_7H_6O_4$ **Protocatechuic acid**		**1.35**	**3805**	**871.80**	**95.89**

Table-4.3b: Identified compounds of GCMS analysis of HFP

Sl. No	IUPAC Name Chemical Formula Common Name	Chemical Structure	RT (min)	Height	Area	Peak Area %
7	2,5-dihydroxybenzoic acid $C_7H_6O_4$ **Gentisic acid**		**2.64**	**60**	**11.93**	**1.31**
8	2,4-dihydroxybenzoic acid $C_7H_6O_4$ **2,4-Dihydroxybenzoic acid** (β-Resorcylic acid)		**3.42**	**157**	**25.42**	**2.80**
9	4-Hydroxy-3-methoxybenzoic acid $C_8H_8O_4$ **Vanillic acid**		**4.53**	**156**	**25.86**	**100**
10	3,4,5-Trihydroxybenzoic acid $C_7H_6O_5$ **Gallic acid**		**0.99**	**327**	**20.06**	**100**
11	(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid $C_{10}H_{10}O_4$ **Ferulic acid**		**5.69**	**128**	**23.34**	**100**
12	4-Hydroxy-3,5-dimethoxybenzoic acid $C_9H_{10}O_5$ **Syringic acid**		**6.23**	**89**	**36.79**	**100**

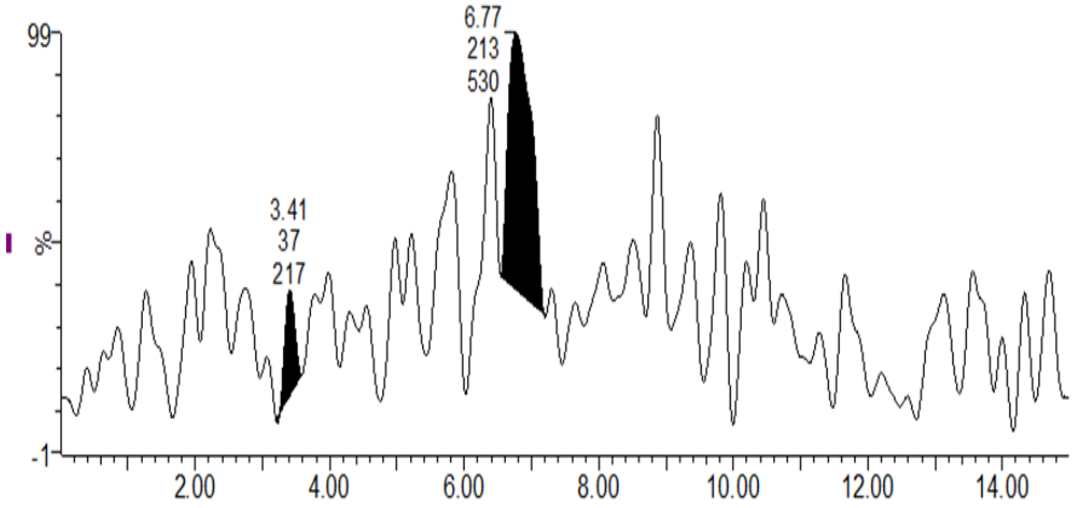


Fig-4.4a - GCMS Chromatogram of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) p-Hydroxybenzoic acid (2) Salisylic acid

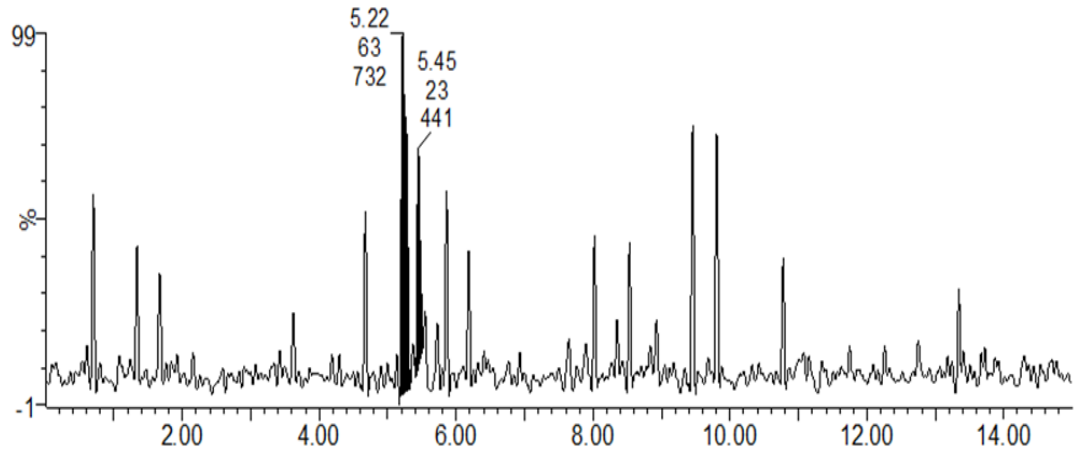


Fig-4.4b– GCMS Chromatogram- of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) o-Coumaric acid (2) p-Coumaric acid

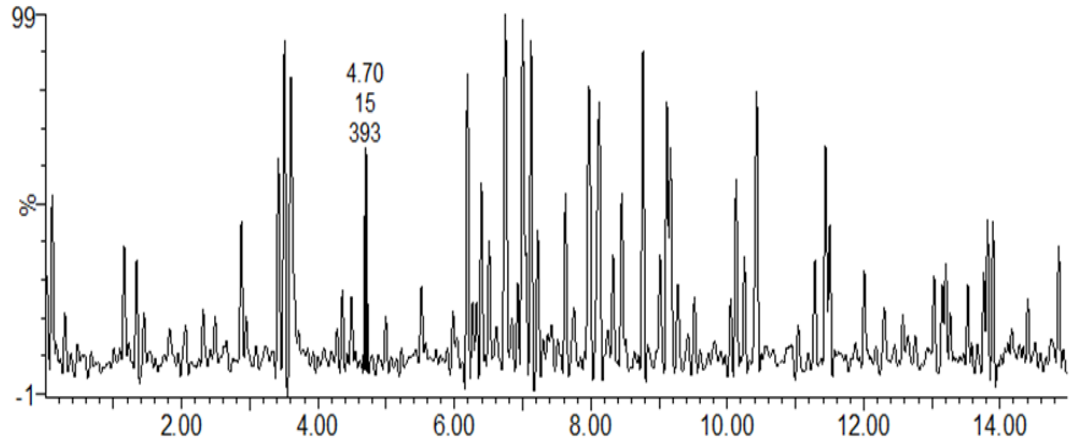


Fig-4.4c– GCMS Chromatogram- of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Caffeic acid

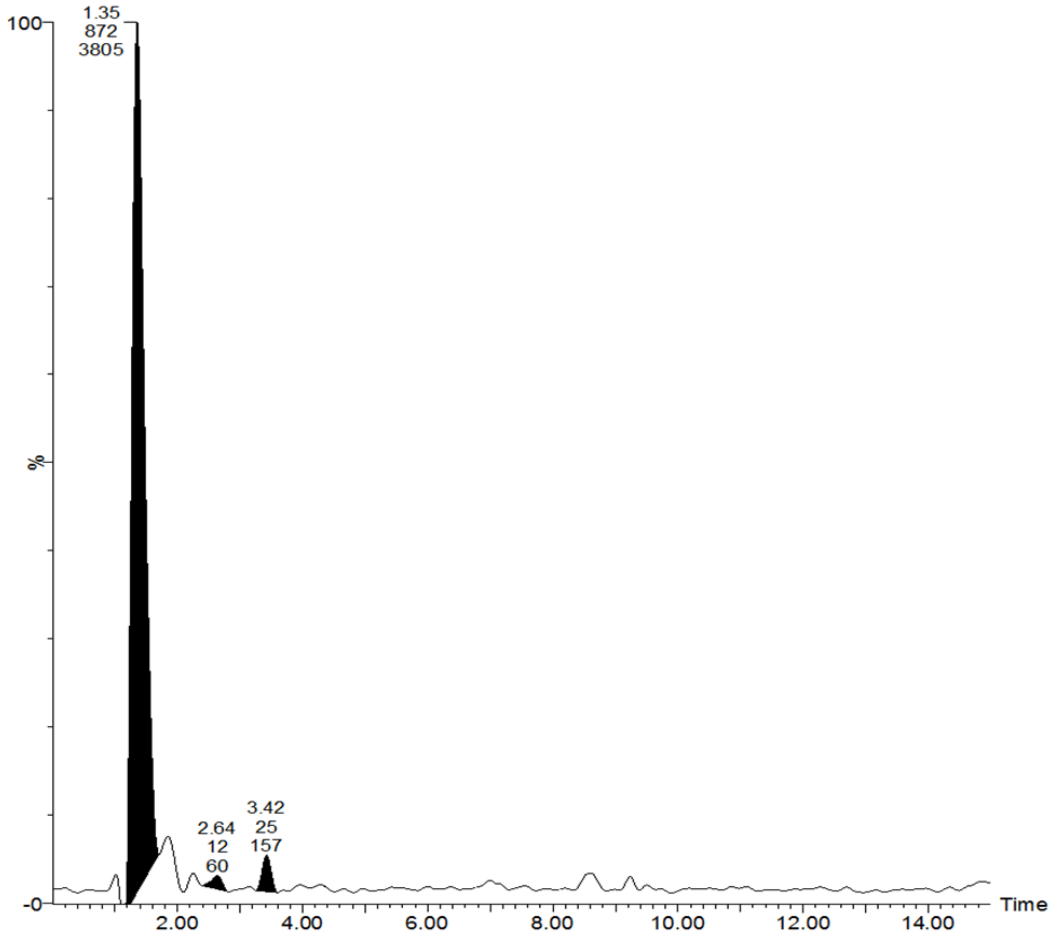


Fig:4.4d– GCMS Chromatogram of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Protocatechuic acid (2) Gentisic acid (3) 2,4-Dihydroxy benzoic acid

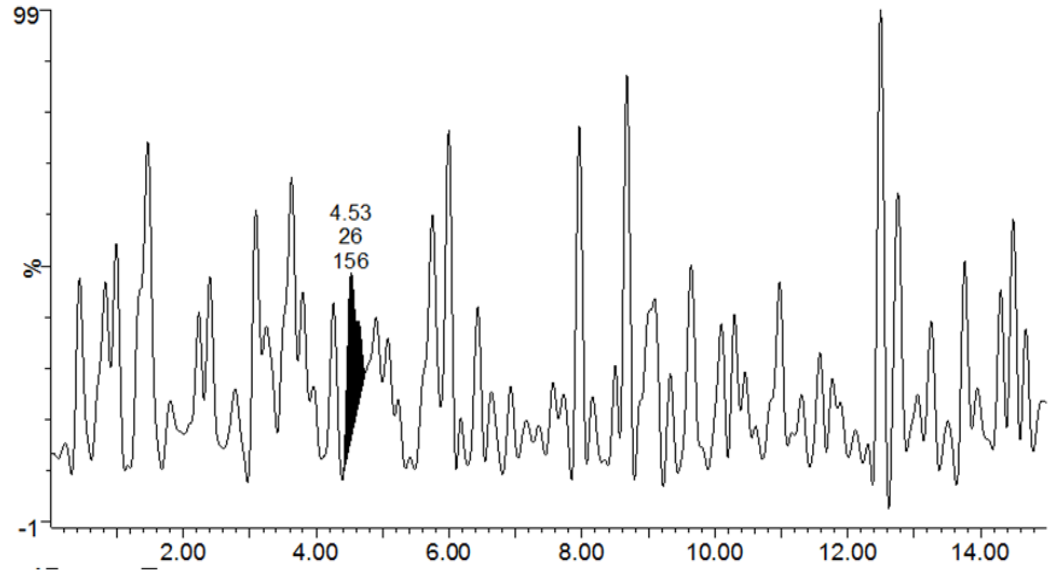


Fig:4.4e– GCMS Chromatogram- of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Vanillic acid

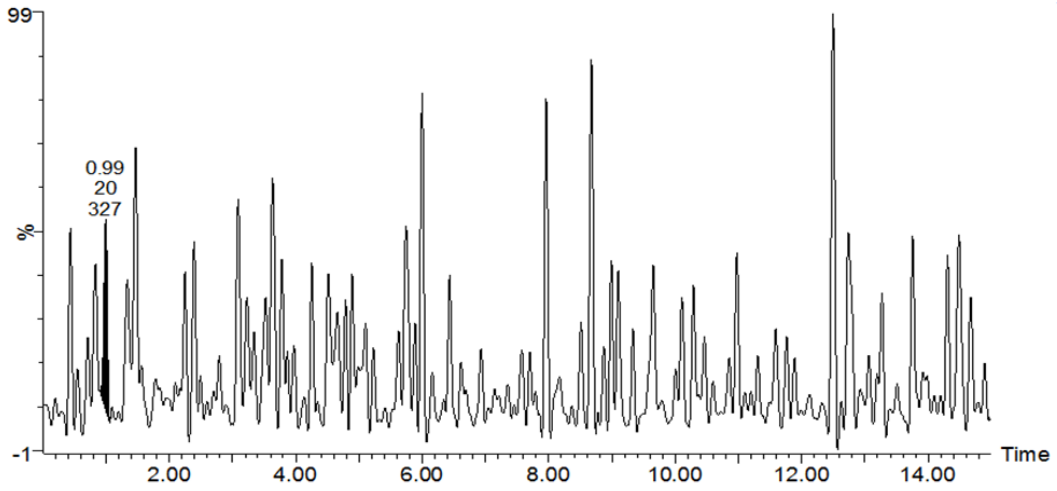


Fig:4.4f– GCMS Chromatogram of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Gallic acid

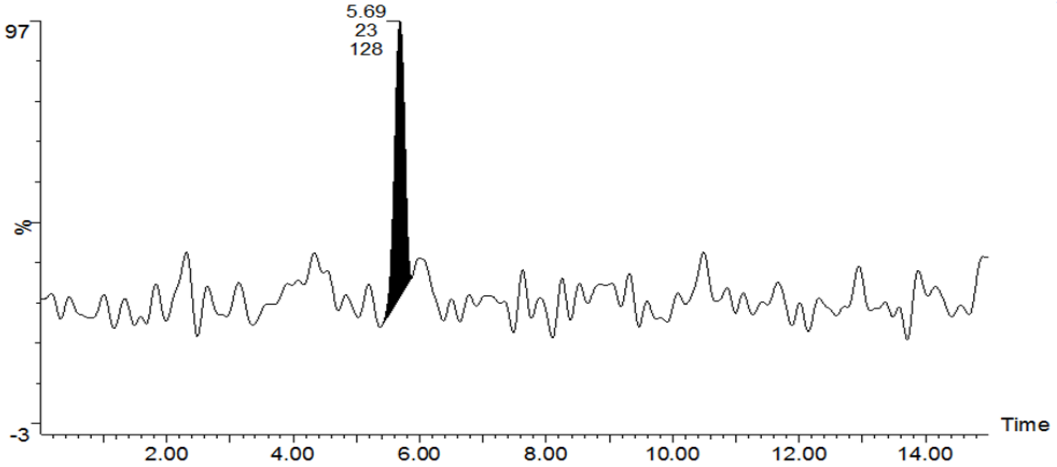


Fig:4.4g– GCMS Chromatogram of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Ferulic acid

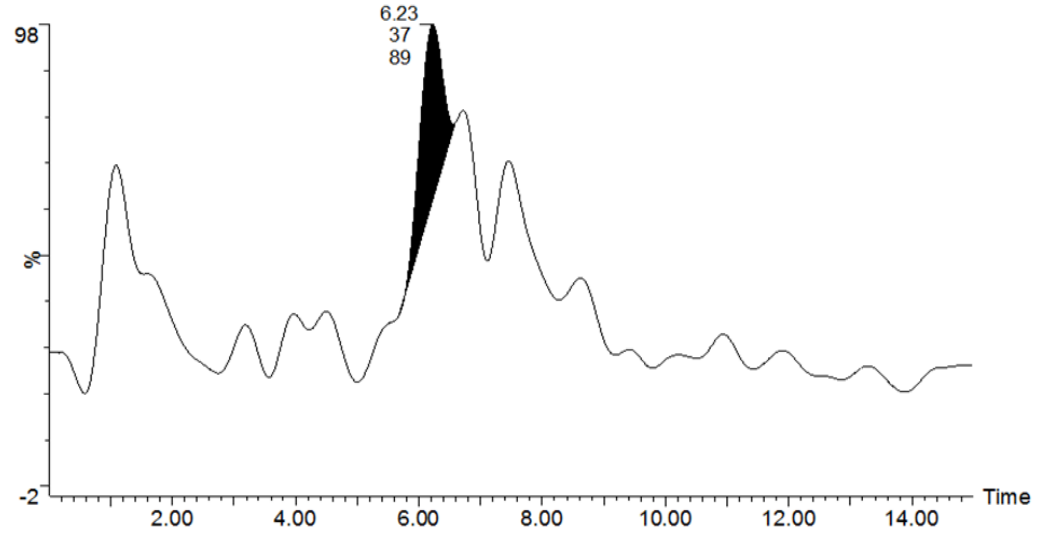


Fig:4.4h– GCMS Chromatogram of *Hodgsonia heteroclita* fruit pulp showing the presence of (1) Syringic acid

The p-Hydroxy benzoic acid; it has an antihyper-glycemic property (Peungvicha et al. 1998) which has also antifungal, anti-mutagenic, antisickling, estrogenic and antimicrobial activities. (Khadem & Marles 2010). Salicylic acid; it has an antihyperglycemic property (Figueroa-Pérez et al. 2015) and has keratolytic, anti-inflammatory, antipyretic, analgesic, antiseptic and antifungal properties for several skin conditions such as dandruff and seborrhoeic dermatitis, ichthyosis, psoriasis, acne etc. (Khadem & Marles 2010). o-Coumaric acid; it is used as antioxidant, hepatotoxicity (Fentem and Fry 1993) and anticancer (Lacy & O'Kennedy 2004). p-Coumaric acid; it has antioxidant (Guleria et al. 2013) and anti cancer properties (Abdel-Wahab et al. 2003). Caffeic acid; it is used as an antidiabetic activity (Jung et al. 2006) and has a powerful antioxidant activity. It increases collagen production and prevents the premature aging. It demonstrates an antimicrobial activity and has promising value in the treatment of dermal diseases (Magnani et al. 2014). Protocatechuic acid (PCA); It has been detected as the most prominent bioconstituents in HFP. PCA has a strong antioxidant property and exerts anti-inflammatory, antihyperglycemic as well as antiapoptotic activities. PCA inhibit chemical carcinogenesis and exert proapoptotic and antiproliferative effects in different cancerous tissues. Moreover, it has antimicrobial activities (Semaming et al. 2015). Gentisic acid; it exhibits an analgesic, anti-inflammatory, antirheumatic, antiarthritic, and cytostatic agent. It inhibits low-density lipoprotein oxidation in human plasma. It is also believed that gentisic acid has an effective role in the anticarcinogenetic activity. 2,4-dihydroxybenzoic acid; has thyroid peroxidase inhibitory effect (Khadem & Marles 2010). Vanillic acid; it has antidiabetic property (Coman et al. 2012). Besides antisickling and anthelmintic activities, it can suppress hepatic fibrosis in chronic liver injury. It is also found to be an inhibitor of snake venom 5'-nucleotidase (Khadem & Marles 2010). Gallic acid; it has an astringent and styptic property. Besides having antineoplastic and bacteriostatic activities, it possesses antimelanogenic and antioxidant properties. It is a potent inhibitor of brush border sucrase and other

disaccharidases in the mammalian intestine. It shows promising results as an anti-HSV-2 (Herpes simplex virus) agent. It has been proposed for the treatment of brain tumours as it suppresses cell viability, proliferation, invasion and angiogenesis in human glioma cells (Khadem & Marles 2010). Ferulic acid; it exhibits a wide range of therapeutic effects against various diseases like- cancer, diabetes, cardiovascular dysfunction and neurodegenerative, inflammatory diseases and in ageing. Besides it has a strong antioxidant property (Srinivasan et al. 2007). Syringic acid has antibacterial and hepatoprotective activities (Khadem & Marles 2010).

4.4 *Invivo* studies

4.4.1 Acute toxicity (LD_{50} dose): After 10-12 hours of the drug administration of *Hodgsonia* fruit pulp (HFP) extract to swiss albino mice, the primary sign of toxicity was noticed with decrease in locomotion and sense of touch. After 18 hours it had noticed as reduced feed intake and prostration. By 24 hours when the animals were examined some of them were already death and others were survived. As per Karber method modified by Aliyu and Nwude (1982) the median lethal dose (LD_{50}) of the hydro-methanolic extract of HFP was revealed 882mg/mL/Kg body weight in mice which was relatively safe for application.

The plant materials may have enough degree of toxicity to consumption which may be fatal to living organisms. For that the orthodox drugs may be unprofessional practice (Chan 2003). So the standardizations of the toxicity of the sample is very much necessary for the effective and relatively safe for application (Lorke 1983).

4.4.2 Effect of HFP Extract on Fasting Blood Glucose (FBG): The antihyperglycemic effects of hydro-methanolic extract of *Hodgsonia* fruit pulp to the fasting blood glucose concentration of the alloxan induced experimental diabetic rats seems as shown in the figure-4.5.

The experimentation of alloxan induced diabetic control (DC) rats showed a significant increase in the fasting blood glucose concentration initially 270 mg/dL and by the six weeks it has been reached 415 mg/dL in comparison to the normal control (NC) blood glucose concentration of 94mg/dL initially to 98mg/dL finally.

The daily treated rats with the groups of low dose (LH, 20 mg/Kg) and high dose (HH, 40 mg/Kg) of methanolic HFP extract seems the fasting blood glucose concentration initially 320.6 mg/dL and 333.3 mg/dL which has been reached to 228.25 mg/dL and 201mg/dL by the six weeks respectively. It means that 45% and 52% reduction of glucose concentration by treating low dose and high dose of HFP extract respectively. On the other hand the experimental groups of diabetic glibenclamide (DG) and diabetic insulin (DI) treated rats initially showed 297 mg/dL and 316.7 mg/dL concentration of blood glucose and by the 42 days it had reached to 217 mg/ dL and 94 mg/dL in six weeks respectively which shows that the significant reduction of blood glucose concentration.

4.4.3 Effect of HFP on Pancreatic Enzymes: The effects of HFP on the pancreatic endogenous enzymatic defensive antioxidant to the alloxan induced experimental diabetic rats exhibited a significant variation. After administration of HFP extract for a period of six weeks the antioxidative enzymes, Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Malondialdehyde (MDA) levels were determined in the pancreas of the experimental rats as depicted in the figure-4.6.

4.4.3.1 Effect on the SOD activity: SOD is an endogenous enzymatic defensive antioxidant which protects the tissues against oxygen free radicals by catalyzing the dismutation of superoxide radicals converting it into the hydrogen peroxide and molecular oxygen (Devasagayam et al 2004). The reactive oxygen species of SOD is effective when its activity is followed by GPx. GPx is involved in detoxifying the hydrogen peroxide (H_2O_2) generated by SOD and other organic hydroperoxide (ROOH) and thus protects the membrane lipid peroxidation (Halliwell 2000). SOD activity in diabetic control (DC) rat was significantly decreased in the pancreas up to 8.91 U/mg protein in comparison to the 18.34 U/mg protein in the normal control (NC) rats. The supplementation of low dose extract and high dose of Hodgsonia extract exhibited 11.03 U/mg protein and 12.32 U/mg protein respectively. It is a significant reclamation of the SOD activity in the pancreas of

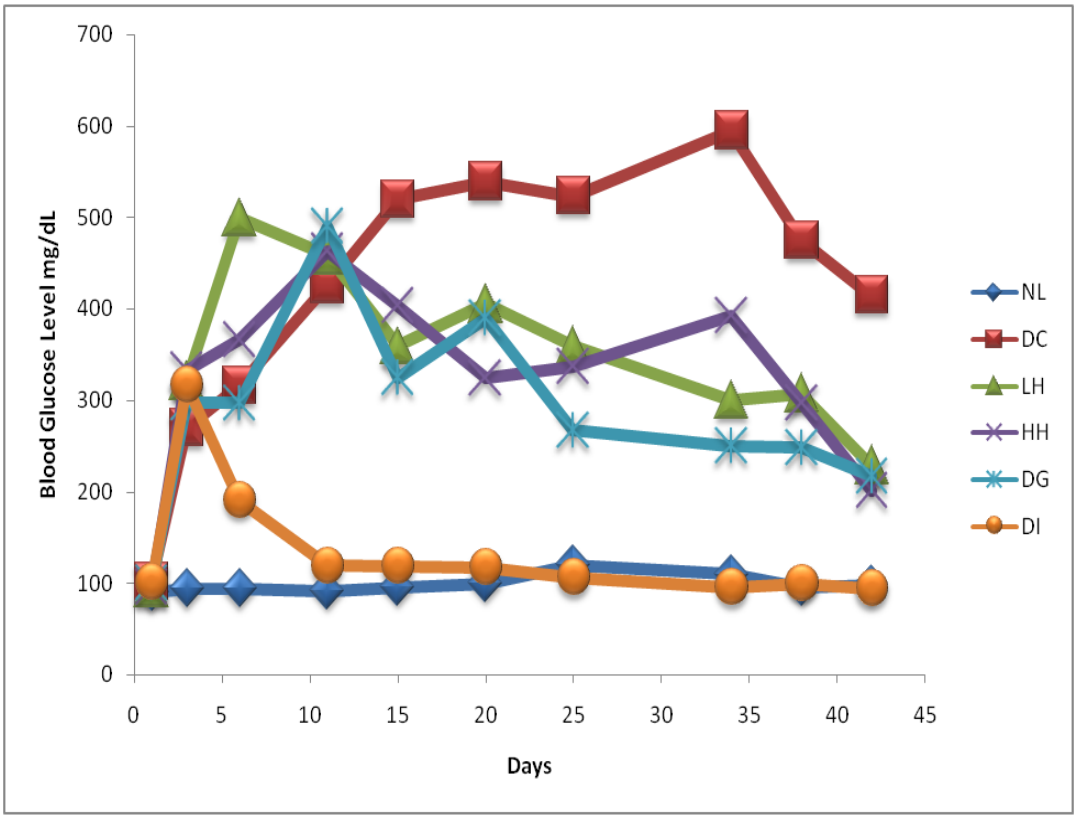


Fig-4.5: Estimation of Fasting Blood Glucose Level (FBGL)

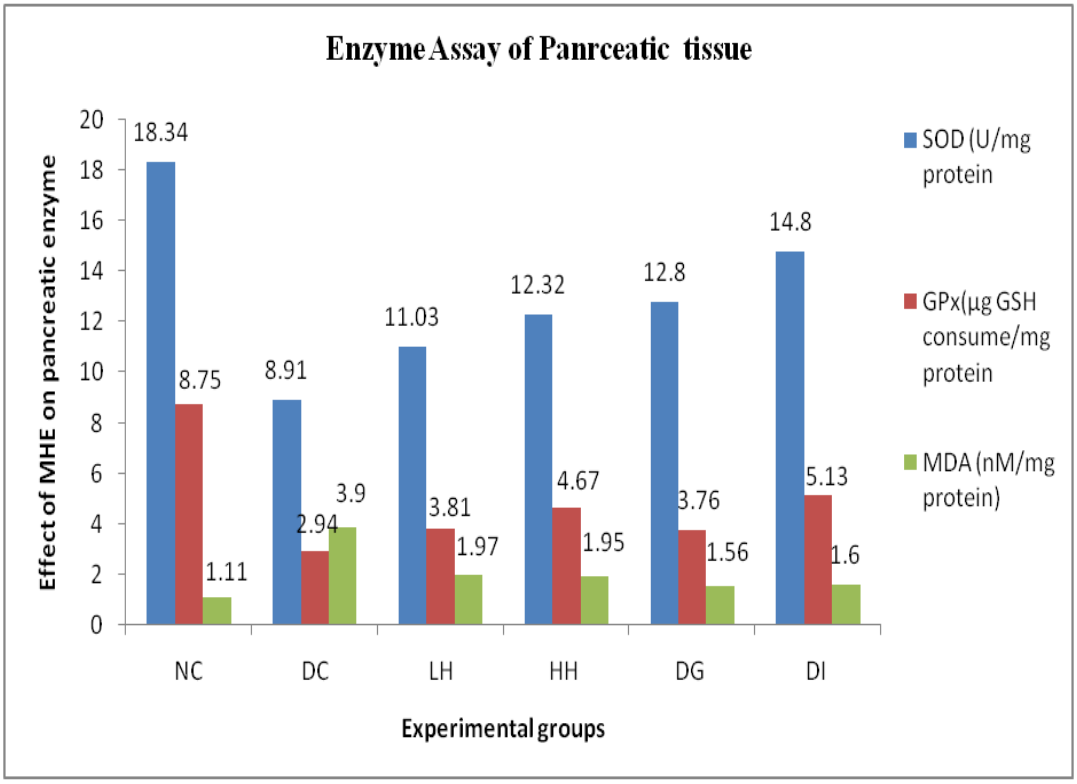


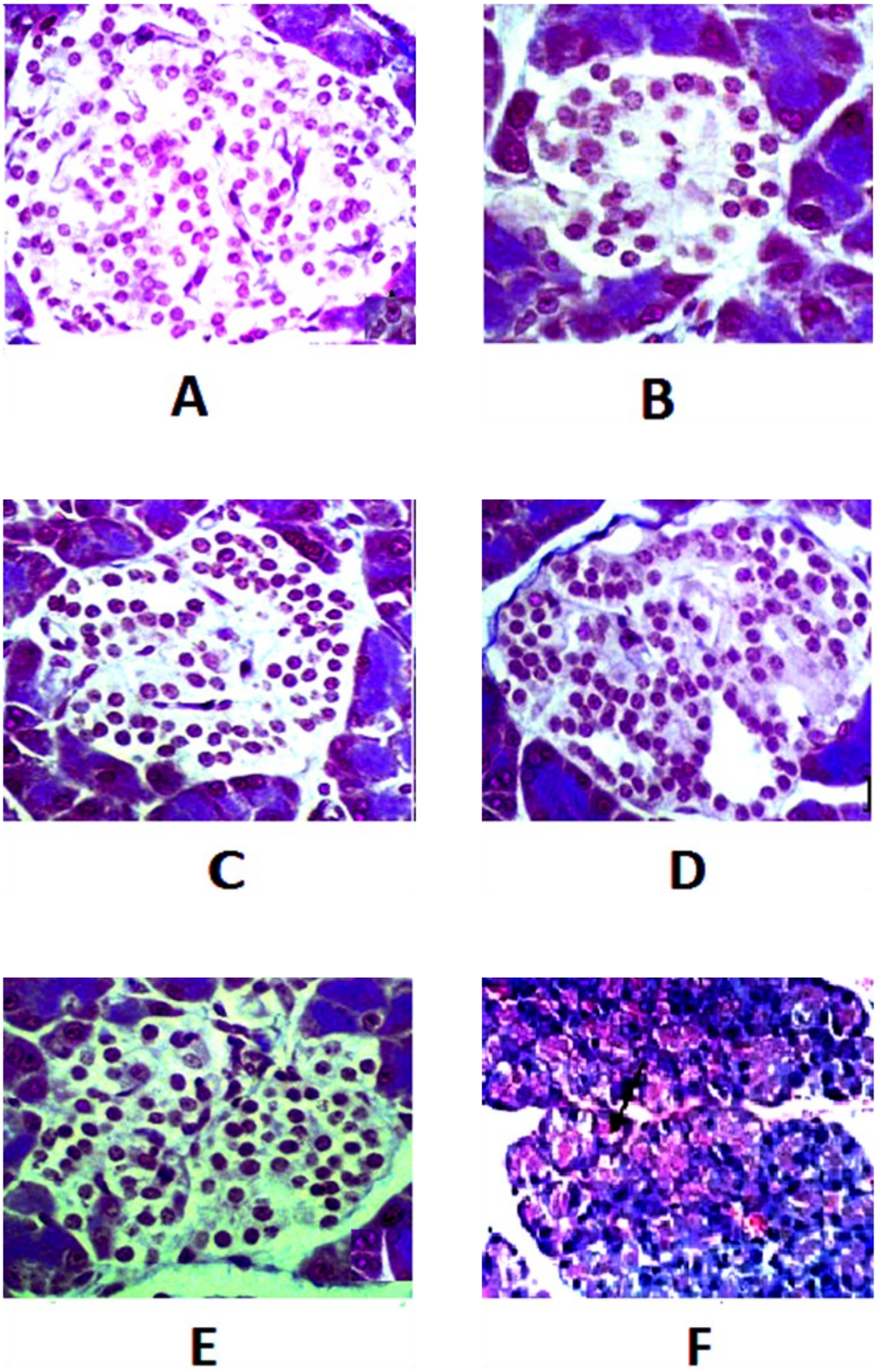
Fig-4.6: The effects of hydro-methanolic extract of *Hodgsonia* fruit pulp on pancreatic enzymatic

experimented rats in comparison to the diabetic control rats. Similar trend was shown in the glibenclamide and insulin treated rats as 12.8 U/mg protein and 14.8 U/mg protein respectively.

4.4.3.2 Effect on the GPx activity: Similarly glutathione (GSH) protects cells from oxidative stress by reducing disulfide bonds of cytoplasmic proteins to cysteines. During this process glutathione is oxidised to glutathione disulfide (GSSG). Glutathione peroxidases (GPx) catalyze the breakdown of hydrogen peroxide and organic hydroperoxides (Liou et al 2010). The GPx activity showed reduced in diabetic control (DC) rats up to 2.94 µg GSH consume/mg protein with respect to the normal control (NC) rats up to 8.75 µg GSH consume/mg protein. The treatments with low dose and high dose of *Hodgsonia* extract exhibited 3.81 µg GSH consume/mg protein and 4.67 µg GSH consume/mg protein respectively. The pancreatic tissue of experimental rats administered with LH and HH dose of HFP extract revealed a significant increase of GPx activity. Glibenclamide and insulin treated rats showed the elevation of the GPx activity up to 3.76 U/mg protein and 5.13 U/mg protein respectively.

4.4.3.3 Effect on the MDA activity: The MDA is one of the final products of polyunsaturated fatty acid peroxidation whose production increases with the increase of free radicals in the cells. MDA level is popularly designated as biomarker of oxidative stress (Goyal et al 2017). In present study the diabetic control (DC) rats showed 3.9 nM/mg protein in comparison to the 1.11 nM/mg protein of normal control (NC) rats which is a significant increase of MDA compared to the normal rats. Supplementation LH & HH dose of HFP extract exhibited 1.97 nM/mg protein and 1.95 nM/mg protein respectively which is the lowered MDA activity compared to the diabetic control rats. The experimental rats treated with the glibenclamide and insulin showed 1.56 nM/mg protein and 1.6 nM/mg protein respectively which is a reduced LPO activity.

4.4.4 Pancreas Histopathology: The haematoxylene and eosin stain of pancreatic tissue of experimental rats revealed the observable variation as



[Where, A= normal control, B= diabetic control, C & D= treated with low and high dose of methanolic *Hodgsonia* extract, E & F= diabetic glibenclamide and diabetic insulin]

Fig-4.7: Photomicrographs of rat pancreas by haematoxylene and eosin stain.

given in the figure-4.7. The supplementation of effective drug reduces the oxidative stress of living body. The reduced pancreatic β-cells induced by the alloxan treatment regenerates not only by the proliferation of pre existing β-cell but also neogenesis from the non β-cells pancreas (Tamura et al. 2015). The experimental rats of diabetic control (DC) group showed degenerated pancreas with the reduction in number and size of β-cells of Islets of Langerhans in comparison to the normal control (NC) group. The groups of rats treated with low (LH) and high dose (HH) of hydromethanolic extract of *Hodgsonia* fruit pulp showed the restoration of the size and number of the β-cell of Islets of Langerhans which were comparable to the glibenclamide treated rats.