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EXTRACTION, PHYTOCHEMICAL SCREENING AND HEPATOPROTECTIVE ACTIVITY OF DACTYLORHIZA HATAGIREA ROOT EXTRACT

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ABSTRACT

Natural products are playing a vital role in health care for decades. Often different sources of natural products, plants have been a source of chemical substance, which serves as drugs in their own right or key ingredients in formulation containing synthetic drugs. Present Investigation deals with hepatoprotective effect of hydroalcoholic extract of roots of Dactylorhiza hatagirea. The plant will be collected from local area of Bhopal, India. The authenticity of the plant was confirmed by botanist. The preliminary phytochemical analysis of the plant extracts will be performed using the standard protocol. Evaluate the hepatoprotective activity of hydroalcoholic extract using Paracetamol induced hepatotoxicity. The present study revealed a significant increase in the marker enzymes like AST, ALT, ALP and serum bilirubin levels, on exposure to Paracetamol, indicating considerable hepatocellular injury. Oral administration of Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) at two different dose levels attenuated the increased levels of the marker enzymes produced by Paracetamol and caused a subsequent recovery towards normalization almost like that of standard silymarin treatment. The results showed that the Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) at different dose levels offer hepatoprotection.

KEYWORDS: natural products, Dactylorhiza hatagirea (Root), Hydro-alcoholic extracts, hepatotoxicity.

INTRODUCTION

Liver diseases have become a major global health challenge and may be triggered by several toxic chemicals, which include chemotherapeutic agents, thioacetamide, carbon tetrachloride, certain antibiotics, excessive alcohol consumption, and pathogenic microbes. Hence, safeguarding a healthy liver is vital for good health and well-being. Despite advances in pharmacology, the demerits associated with synthetic drugs have outshone the merits. Treatment of liver diseases based on modern medical principles is becoming ineffective and also associated with adverse effects of long-term use, in addition to prohibitive costs in developing countries.

Thus, exploring medicinal plants which are easily available and cheap and do not involve strenuous pharmaceutical production processes appears to have gained worldwide attention as alternative therapeutic agents for the diseases. Consequently, emphasis has been placed on folkloric herbs with high efficacy, low toxicity, and cost-effectiveness.[1]

The liver is the largest solid organ, the largest gland and one of the most vital organs that functions as a centre for metabolism of nutrients and excretion of waste metabolites. Its primary function is to control the flow and safety of substances absorbed from the digestive system before distribution of these substances to the systemic circulatory system.[2] A total loss of liver function could leads to death within minutes, demonstrating the liver‘s great importance, in view of this, this study was undertaken to review the physiology of the liver with a view to keep it functioning at its optimum and maintaining good health so as to avoid liver damages such as fatty liver, liver fibrosis and cirrhosis.[3]

Hepatotoxicity

Hepatotoxicity implies chemical-driven liver damage. Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the organ. Other chemical agents, such as those used in laboratories and industries, natural chemicals (e.g., microcystins) and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins. More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Chemicals often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. More than 75% of cases of idiosyncratic drug reactions result in liver transplantation or death.[4]

Hepatotoxicity inducing agents

Many xenobiotics like chemicals, drugs, house hold things, herbs and environmental factors are well-known to induce hepatotoxicity. Most significant for xenobiotic- induced liver injury, the centrilobular (zone-3) hepatocytes are the 1st sites of haemoprotein P450 accelerator activity, which regularly makes them at maximum risk of xenobiotic-induced liver injury. CCl4, N-nitrosodiethylamine, Acetylaminofluorene, Galactosamine, d-Galactosamine/ Lipopolysaccharide, TAA, Antitubercular drugs, PCM, Arsenic etc.[5]

Ayurvedic medicines

Ayurvedic medicines mainly based on plants enjoy a respective position today, especially in the developing countries, where modern health services are limited. Safe effective and inexpensive indigenous remedies are gaining popularity among the people of both urban and rural areas especially in India and China.[6] Information from ethnic groups or indigenous traditional medicines has played vital role in the discovery of novel products from plants as chemotherapeutic agents. Herbal medicines have been main source of primary healthcare in all over the world. From ancient times, plants have been catering as rich source of effective and safe medicines.[7] About 80 % of world populations are still dependent on traditional medicines. Herbal medicines are finished, labeled medicinal products that contain as active ingredients, aerial or underground part of plants or other plant materials, or combination thereof, whether in the crude state or as plant preparations. Medicines containing plant materials combined with chemically defined active substances, including chemically defined isolated constituents of plants are not considered to be herbal medicine.[8]

MATERIALS AND METHODS

Plant material collection

The plants have been selected on the basis of its availability and Folk use of the plant. Root of Dactylorhiza hatagirea were collected from local area of Bhopal in the month of November, 2021. Drying of fresh plant parts were carried out in sun but under the shade. Dried root of Dactylorhiza hatagirea were preserved in plastic bags and closed tightly and powdered as per the requirements.

Physicochemical evaluation of plant

The use of herbal medicines continues to expand rapidly across the world. Many people now take herbal medicines or herbal products for their health care in different national health-care

settings. Authentication and standardization are prerequisite steps while considering source materials for herbal formulation in any system of medicine.

Determination of Total ash

About 2 gm accurately weighed powdered drug was incinerated in a silica dish at a temperature not exceeding 450° C until free from carbon. It was then cooled and weighed. The percentage w/w of ash with reference to the air-dried drug was calculated.[9]

Determination of Acid insoluble ash

Ash is boiled with 25 ml dilute HCL (6N) for five minutes. The insoluble matter collected on an ash less filter paper, washed with hot water and ignited at a temperature not exceeding 4500C to a constant weight.[10]

Determination of Water-soluble ash

Ash is dissolved in distilled water and the insoluble part collected on an ash less filter paper and ignited at 4500C to constant weight. By subtracting the weight of insoluble part from that of the ash, the weight of soluble part of ash is obtained. Percentage of water soluble ash was calculated with reference to the air dried drug.[11]

Determination of Sulphated ash

2g of powdered material was taken in crucibles and ignited at 4500C in a muffle furnace until the material gets thoroughly charred. The crucibles along with ash are taken out in desiccators and cooled. 1ml H_2SO_4 was added to each crucible in order to moisten the residue. Heat gently until white fumes was no longer evolved and ignites at 8000C until black particles were disappeared. The crucibles are removed from the muffle furnace and transferred to desiccators, cooled and weighed to give the sulphated ash content.[9]

Determination of Loss on drying

5 g of the powdered sample was taken in a tarred weighing bottle and weighed accurately. It was dried at 105°C for 5 hours and allowed to cool in desiccators and weighed. The drying and weighing was continuous at 1-hour interval until difference two successive weighing. When the weight of sample became constant, the loss in weight and the percentage of loss on drying were calculated.[12]

Determination of Foreign matter

The 50 gm drug sample was spread in a thin layer and the pieces of foreign matter were sorted out by visual examination. The powder of foreign matter was sifted through a 250-micron sieve. All portions of the foreign matter were pooled and weighed.[13]

Extraction procedure

Following procedure was adopted for the preparation of extracts from the shade dried and powdered herbs.[14,15]

Defatting of plant material

Root of Dactylorhiza hatagirea were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place.

Extraction by maceration process

46.85 gm of dried powdered root of Dactylorhiza hatagirea has been extracted with Hydroalcoholic solvents (Ethanol 70%) using maceration process for 48 hrs, filtered and dried using vacuum evaporator at 400C.

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

$$\text{Percentage yield} = \frac{Weight\ of\ Extract}{Weight\ of\ powder\ drug\ Taken} \times 100$$

Phytochemical Screening

Phytochemical examinations were carried out for all the extracts as per the standard methods

In vivo hepatoprotective studies.

Selection and maintenance of animals.[16]

Healthy adult male albino rats of Wistar strain weighing between 180-220 g were used for the screening of hepatoprotective activity of the plant extracts. The animals were housed in polypropylene cages in adequately, well ventilated room and maintained under standard environmental conditions (22-28°C, 60-70% relative humidity, 12 h dark/light cycle). The animals were fed with standard rat feed pellets and water ad libitum (Aquaguard filter water). The study was approved by the institutional animal ethics committee.

Acute toxicity studies

Acute oral toxicity study was performed as per OECD- 423 guidelines (acute toxic class method). Wistar rats (n = 6) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for a overnight providing only water, after which the extracts were administered orally at the dose level of 5 mg / kg body weight by intragastric tube and observed for 14 days. No mortality was observed in any group of animal, then the same dose was repeated again to confirm the toxic dose, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg / kg body weight.[17]

Preparation of the drug for the experimental study

The extracts and the standard drugs were administered in the form of suspension in water with 1% sodium carboxymethyl cellulose as suspending agent.

Preparation of the standard

Silymarin, a known hepatoprotective agent was used as the reference for comparison at a dose of 25 mg/kg body weight in 0.3% CMC.

Experimental design

The animals were divided into 5 groups of six rats each.

Group I: Animals served as normal control and received distilled water for seven days.

Group II: Animals orally received paracetamol (500 mg/kg body weight) for seven days.

Group III: Animals received 200 mg/kg body weight of standard drug silymarin and 500 mg/kg body weight of paracetamol for seven days and served as standard control.

Group IV: Animals received 500 mg/kg body weight of paracetamol dissolved in glucose water orally along with 200 mg/kg body weight of Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) for seven days orally.

Group IV: Animals received 500 mg/kg body weight of paracetamol dissolved in glucose water orally along with 300 mg/kg body weight of Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) for seven days orally.

Isolation of blood serum for biochemical studies

Blood was collected from jugular veins and centrifuged (3000 rpm for 10 min) to obtain serum. The serum was used for marker enzyme estimation. Assay of aspartate aminotransferase (AST or SGOT)

Asparate amino transferase (AST) catalyzes the following reaction. 2-oxoglutarate + L-aspartate ↔ glutamate + oxaloacetate oxaloacetate + NADH + H+ ↔ malate + NAD+

The rate of NADH consumption was measured photometrically at 540 nm and is directly proportional to the AST activity in the sample, AST level in serum is expressed as U/L.

Estimation of aspartate aminotransferase

Aspartate aminotransferase was estimated by the method of (King J).[18]

Reagents

1. Phosphate buffer 0.1 M, pH 7.5.
2. Substrate: 1.33 g of DL-aspartic acid and 15 mg of 2-oxoglutarate were dissolved in 20.5 ml of 1 N sodium hydroxide and made up to 100 ml with buffer.
3. 0.02% 2,4-dinitrophenyl hydrazine (DNPH): 20 mg of DNPH in 100 ml of 1 N hydrochloric acid.
4. 0.4 N Sodium hydroxide.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1 µm of pyruvate/ml.

Procedure

1.0 ml of buffered substrate was incubated at 37°C for 10 min. Then 0.2 ml of enzyme was added and mixture was incubated at 37°C for 1 hr. To the control tubes enzyme was added after the reaction and it was arrested by the addition of 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 30 min. A set of standard pyruvate solution was also treated in a similar manner. Then 5.0 ml of sodium hydroxide was added. The colour developed was read at 540 nm.

The enzyme activity is expressed as U/L.

Assay of alanine aminotransferase (ALT or SGPT)

Alanine aminotransferase (ALT) catalyzes the following reactions. 2–oxoglutarate + L – alanine ↔ glutamate + pyruvate

Pyruvate + NADH + H+ ↔ Lactate + NAD+

The rate of NADH consumption was measured photometrically at 540 nm and is directly proportional to the ALT activity in the sample. Alanine aminotransferase level in serum is expressed as U/L.

Estimation of alanine aminotransferase Alanine aminotransferase was assayed by the method of (King J)[18]

Reagents

1. Phosphate buffer 0.1 M, pH 7.5.
2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 ml of buffer, 0.5 ml of 1 N sodium hydroxide was added and made up to 100 ml with phosphate buffer, pH 7.5.
3. 0.02% 2,4-dinitrophenyl hydrazine (DNPH): 20 mg of DNPH in 100 ml of 1 N hydrochloric acid
4. 0.4 N sodium hydroxide.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 µm of pyruvate/ml.

Procedure

1.0 ml of substrate was incubated at 37°C for 10 min. Then 0.2 ml of enzyme solution was added. The tubes were incubated at 37°C for 30 min. To the control tubes enzyme was added after arresting the reaction with 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 20 min. Then 5.0 ml of 0.4 N sodium hydroxide was added and then the colour developed was read at 540 nm. The enzyme activity is expressed as U/L.

Assay of alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) catalyzes the following reaction.

4-nitrophenyl phosphate + H2O ↔ phosphate + 4-nitrophenolate

The rate of increase in 4-nitrophenolate was determined photometrically at 640 nm and is directly proportional to the ALP activity in the sample. Alkaline phosphate level in serum is expressed as U/L.

Estimation of alkaline phosphatase

Alkaline phosphatase was assayed by the method of (King J)[18]

Reagents

1. Carbonate-bicarbonate buffer 0.1 M, pH 10: 6.36 g of sodium carbonate and 3.36 g of sodium bicarbonate were dissolved in 1000 ml of water.
2. Substrate 0.1 M: 254 mg of disodium phenyl phosphate was dissolved in 100 ml of water.
3. Magnesium chloride 0.1 M: 406 mg of magnesium chloride was dissolved in 20 ml of water.
4. 15% sodium carbonate: 15 g of sodium carbonate was dissolved in 100 ml of water.

5. Folin's phenol reagent: One volume of Folin’s reagent was diluted with two volumes of distilled water just before use.
6. TCA 10%
7. Standard: 10 mg of phenol was dissolved in 100 ml of water

Procedure

The mixture containing 1.5 ml buffer, 1.0 ml substrate and 0.1 ml of magnesium chloride were pre-incubated at 37°C for 10 min. Then 0.1 ml of enzyme was added and incubated at 37°C for 15 min. The reaction was arrested by 1.0 ml of 10% TCA. Control without enzyme was also incubated and the enzyme was added after the addition of TCA solution. Then 1.0 ml of sodium carbonate and 0.5 ml of Folin's phenol reagent were added. After 10 min the blue colour developed was read at 640 nm.

The enzyme activity is expressed as U/L.

Assay of total protein (TP)

Proteins and peptides, in contrast to other nitrogen containing compounds (e.g. creatinine, urea and uric acid) produce a violet coloured complex with copper ions in dilute alkaline solution. The so called biuret reaction is particularly easy to carry out giving reproducible results. The absorbance of the colour complex is directly proportional to the protein concentration in sample materials. The absorbance of the sample and standard was measured against the biuret regent and the absorbance of the blank against distilled water at 640 nm. Total protein level in serum is expressed as mg/dL.

Estimation of protein

Protein was estimated by the method of (Lowry OH)[19]

Reagents

1. Alkaline copper reagent
2. Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate. 50 ml of solution A was mixed with 1 ml of solution B just before use.
3. Folin’s phenol reagent: One volume of Folin’s reagent was diluted with two volumes of distilled water just before use.
4. Standard bovine serum albumin (BSA): 20 mg of BSA was dissolved in 100 ml of distilled water. Few drops of sodium hydroxide (alkali) were added to aid complete

dissolution of BSA and to avoid frothing; it was allowed to stand overnight in a refrigerator.

Procedure

100ml of 10% homogenate was diluted to 1 ml with water. From this diluted samples 0.1 ml was made up to 1 ml with water. Standards were taken and made up to 1 ml with water. 1 ml water was used as blank. To all tubes 4.5 ml of alkaline copper reagent was added and kept at room temperature for 10 min. Then 0.5 ml of Folin's phenol reagent was added and the colour developed was read after 20 min at 640 nm. Protein content is expressed as mg/dl of fresh or wet tissue.

Estimation of total bilirubin

Total bilirubin was estimated by the method of (Malloy HT)[20]

Reagents

1. Absolute methanol
2. HCl, 1.5% v/v with water
3. Diazo reagent: Solution A: About 1 gm of sulphanilic acid was dissolved in 15 ml of concentrated HCl and made upto 1 litre with water. Solution B: About 0.5 gm of sodium nitrate was dissolved in water and made upto 100 ml. Prepared freshly before use by adding 0.3 ml of solution B to 10 ml of solution A.

4. Standard solution of bilirubin

Prepared a solution containing 10 mg of bilirubin per 100 ml of CHCl3. Procedure 10 ml of serum was taken and 1.8 ml of distilled water was added. To the solution, 0.5 ml of diazo reagent was added. Finally, 2.5 ml of methanol was added and stand for 30 min. Absorbance was read in the colorimeter using a yellow green filter or set upto 540 nm. The amount of total bilirubin was calculated and expressed in terms of mg/dl.

Results of Physicochemical evaluation

The physico-chemical evaluation of drugs is an important parameter in detecting adulteration or improper handling of drugs. The present work assists the credit of the plant material for further investigation and form an important aspect of drug studies.

S. No.	Parameter	*Dactylorhiza hatagirea* (w/w)
1	Total ash	8.5%
2	Acid insoluble ash	1.25%
3	Water-soluble ash	3.4%
4	Sulphated ash	4.5%
5	Loss on drying	0.25%
6	Foreign matter	0.50%

Determination of percentage yield

All the obtained extracts were dried and weighed. The percentage yield of each plant was calculated as per standard method. The weighed extract of each plant drug was stored in desiccators for further use. The yields were found to be 10.50g (3.5% w/w of crude drug) of petroleum ether extract with semisolid mass of brown colour, 18.10g (7.61% w/w of crude drug) of (Hydroalcoholic) extract with Orange-Black colour for Dactylorhiza hatagirea. Obtained results were recorded in table.

Table: Extractive values obtained from Dactylorhiza hatagirea extract.

S. No.	Solvents	Color of extract	Yield	% Yield
1	Petroleum ether	Brown	10.50g	3.5%
2	Hydroalcoholic	Orange-Black	18.01g	7.61%

Results of *in vivo* hepatoprotective studies Biochemical studies

The Hydro-alcoholic extracts of *Dactylorhiza hatagirea* (Root) was evaluated for its hepatoprotective activity against Paracetamol induced hepatic damage. Healthy adult male albino rats of Wistar strain weighing 180-220 g were used for the study. Silymarin was used as positive control. The plant extracts at two different dose levels (200, 300 mg/kg, po), showed significant hepatoprotective activity as evidenced by an alteration in the serum enzyme levels.

The effect of Hydro-alcoholic extracts of *Dactylorhiza hatagirea* (Root) at both the dose levels on marker enzymes in serum against Paracetamol induced hepatotoxicity were shown in Table 7.7. Liver damage induced by Paracetamol significantly increased the marker enzymes like AST, ALT and ALP in serum ($P<0.05$). Oral administration of the plant extract of *Dactylorhiza hatagirea* (Root) significantly decreased the level of marker enzymes AST, ALT and ALP ($P<0.01$) in serum. The total bilirubin level was significantly increased ($P<0.05$) in Paracetamol treated animals. The Hydro-alcoholic extracts of *Dactylorhiza hatagirea* (Root) treated animals showed a significantly lower bilirubin level in serum. The total protein level in serum was considerably reduced in Paracetamol toxicity. The The

Hydro- alcoholic extracts of *Dactylorhiza hatagirea* (Root) treated animals significantly increased (P<0.05) the total protein level in serum.

In the assessment of liver damage by Paracetamol, the determination of enzyme levels is largely used. The changes observed with viral hepatitis are also seen in Paracetamol administration.[20] Hence Paracetamol induced livertoxicity was chosen as the experimental model. The ability of the liver protectivedrugs to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which had been disturbed by the hepatotoxin, is the index of its protective effect.[21] The enzymes like AST and ALT are cytoplasmic in origin and necrosis or membrane damage releases the enzymes into circulation and therefore can be measured in the serum. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver.[22] In addition, destruction of hepatic cells causes an elevation in the serum levels of acid phosphatase (ACP) and bilirubin. ACP is localized almost exclusively in the particles and its release parallels that of lysosomal hydrolases. Increase in the serum level of ALP is due to increased synthesis, in the presence of increasing biliarypressure.

Table 7.7: Effect of the extract on serum transaminases, alkaline phosphatase, total protein and bilirubin on control and experimental animals.

Group	Drug and dose (mg\kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (mg/dl)	TB (mg/dl)
I	Normal	91.35± 2.23*	57.33 ± 7.98*	72.67 ±6.41*	109.21± 7.16*	2.03 ± 0.08*
II	Paracetamol induced hepaticdamage(500 mg/kg b.Wt)	160.98±13.23*	168.93±8.47*	132.80±12.30*	194.51±10.78*	4.82 ± 1.10*
III	Paracetamol(50 0 mg/kg b.Wt) + Silymarin(200 mg/kgb.Wt)	101.08± 9.82**	68.83±7.65**	74.11± .35**	116.38± .58**	2.07 ±0.85**
IV	Paracetamol(50 0 mg/kg b.Wt) + Dactylorhiza hatagirea (200 mg/kg b.Wt)	106.00 ± 8.03*	62.00± .52**	84.12± .66**	98.69± 13.08*	2.43 ±0.45**
V	Paracetamol(50 0 mg/kg b.Wt) + Dactylorhiza hatagirea (300 mg/kg b.Wt)	108.00 ± 6.02*	66.00± .33**	89.12± .42**	102.58±14.07*	2.88 ±0.56**

Values are expressed as mean ± SEM; n = 6 in each group. A P <0.01 when compared to control; b P <0.01, cP<0.05 and d P>0.05 when compared to.

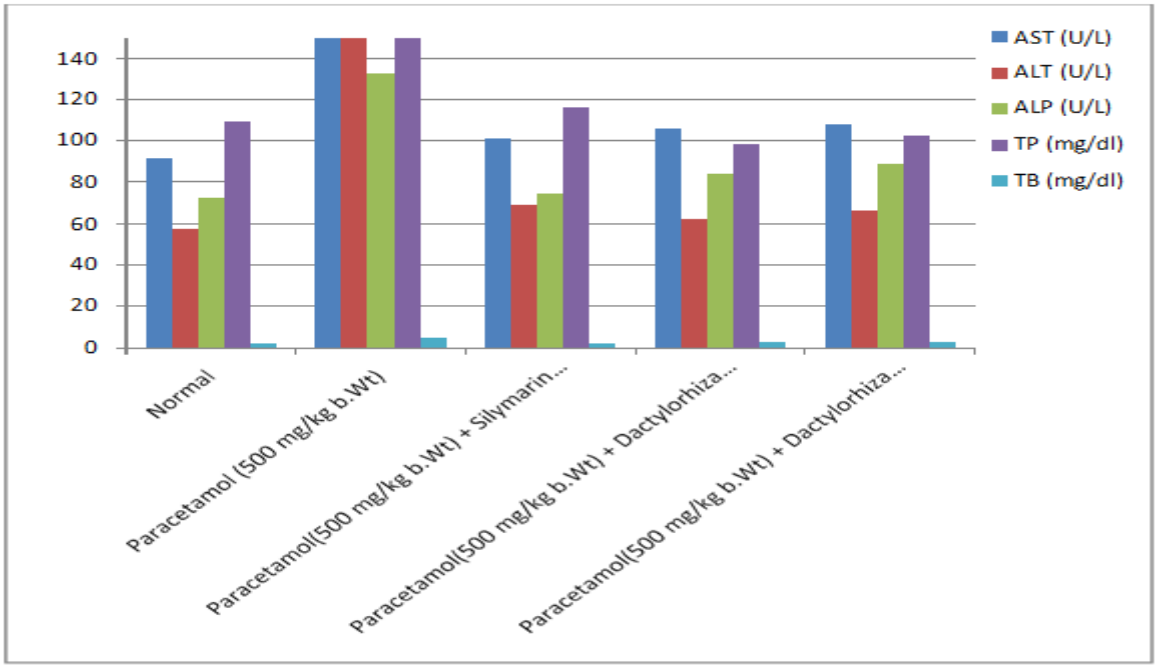

Figure: Biochemical parameters of AST, ALT, ALP, TP, TB

SUMMARY AND CONCLUSION

The present study revealed a significant increase in the marker enzymes like AST, ALT, ALP and serum bilirubin levels, on exposure to Paracetamol, indicating considerable hepatocellular injury. Oral administration of Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) at two different dose levels attenuated the increased levels of the marker enzymes produced by Paracetamol and caused a subsequent recovery towards normalization almost like that of standard silymarin treatment. The decreased total protein level observed in the rats treated with Paracetamol may be due to the decrease in the number of hepatocytes which in turn may result in decreased hepatic capacity to synthesis protein.

On administration of Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) showed significant increase in total protein level, which indicates the increase in hepatocyte levels, accounting for its hepatoprotective effect. The subsequent recovery towards normalization of these enzymes strongly suggests the possibility of the extracts being capable of conditioning the hepatocyte so as to cause accelerated regeneration of parenchyma cells. The results showed that the Hydro-alcoholic extracts of Dactylorhiza hatagirea (Root) at different dose levels offer hepatoprotection.

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Conflict Of Interest

No conflicts of interest is declared by the author.

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