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HEPATOPROTECTIVE ACTIVITY OF ETHANOLIC ROOT EXTRACT OF POMEGRANATE (*Punica granatum*) IN RABBITS AGAINST HEPATITIS

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Abstract

Background:
The natural products which have convey into being direct medicinal relevance as drug entity many others can function chemical models or templates for the design, synthesis, and semi synthesis of new substances for disease remedy of person.

Objective:
To determine the phytochemicals and test the hepatoprotective activity of Punica granatum in CCl4 triggered liver cells in rabbits with the aid of biochemical evaluation of ALT and AST of plant extract to govern the hepatitis disorder.

Methods:
Rabbits were divided into four groups. Each group consists of six animals (n=6) as follow:
Normal Group: Normal and healthy rabbits.
Control Group: The liver damaged rabbits that received CCl_4 + olive oil i.p. for 7 days.
Treatment Group-I: Rabbits that received CCl_4 + olive oil for 7 days and then treated with extracts of Punica granatum at amount of 500mg orally for 10 days.
Treatment Group-II: Rabbits received CCl_4 the same as in Group-I, but extracts of Punica granatum at amount of 1000mg was given orally for 10 days.
The blood samples were collected and allowed to coagulating for 30 min. at 37 °C. The clear serums were separated at 3000 rpm for 10 min. and subjected to the estimation of biochemical analyses, asparate amino-transferase (AST) and alanine amino-transferase (ALT).

Results:
The result indicated that the root extract of Punica granatum was useful to induced the liver cells in the rabbits to protect against the harmful diseases like hepatitis as compared to its low concentration dose by Least Significant Difference (LSD) test.

Conclusion:
Punica granatum root extract used in hepato protective activity. There is a beat of global understanding about the significance of many plants to protect the liver damaged by hepato-toxins.

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INTRODUCTION

Medicinal plant life has fashioned the basis of traditional device of drugs that are in existence for thousands of years. Plants are the items of nature used to deal with number of human diseases (1). Recently, there was an interest in "rediscovering herbal products" (2) because of a fast increase within the fee of infections, antibiotic resistance in microorganisms and additionally because of side effects of synthetic drugs. Contrary to the artificial capsules, antimicrobials of plant beginning are not associated with aspect consequences and have an tremendous therapeutic potential to heal many infectious sicknesses (1).

The pomegranate, *Punica granatum* L, is a fruit bearing small tree about 5 to 8 meter. It is commonly found in Northern Hemisphere, the fruit is usually in season from September to February, and in the Southern Hemisphere from March to May. As intact arils or juice, pomegranates are used in cooking, baking, meal garnishes, juice blends, smoothies, and alcoholic beverages, including cocktails and wine. The pomegranate originated in the region of present day-day Iran and has been cultivated since ancient instances throughout the Mediterranean location and northerly India. It became added into America (Spanish America) in the past due sixteenth century and California by Spanish settlers in 1769. Today, it's miles widely cultivated throughout the Middle East and Caucasus location, north Africa and tropical Africa, the Indian subcontinent, Central Asia, and the drier parts of southeast Asia. It is likewise cultivated in components of California and Arizona. In current years, it has end up more commonplace in the industrial markets of Europe and the Western Hemisphere

The pomegranate has following main parts which are seed, juice, peel, leaf, flower and root bark, every part has its own importance and used in different recipes, formulations, and cosmetics due to presence of essential compounds which are scientifically supported to health benefits such as in arteriosclerosis, to control cholesterol level and in many cancers' prevention. The are several components which are used as raw materials for tannins, dyes, and alkaloids (3, 4, 5). The physicochemical properties of the pomegranate depend on the type of cultivar, growing area, and climate, the fruit's level of adulthood and production systems (6, 7, 8, 9, 10, 11).

The half weight of the whole fruit weight corresponds to the peel about 50 %, that's an important source of bioactive compounds which include phenolics, flavonoids, ellagitannins (ETs), and proanthocyanidin compounds (12), minerals (12), and complicated polysaccharides (13). The mean full difference in chemical compounds such as phenolic compounds, water-soluble nutrients and minerals of pomegranates had been reported in different research articles (14, 15).

The worldwide surveys reported that many tunors get up from sites of contamination and chronic infections [16, 17]. The inflammatory reaction brings about oxidative changes in tumour infection sites and help in tissue repair and their regeneration, response due to harmful infectious or non-infectious disease such as microbial infections (18, 19).

Most of the researcher papers indicated that (20, 21) pomegranate flowers have abundant ellagitannins, which commonly used in Unani and Ayurvedic drugs for the treatment of diabetes. The most powerful effect of pomegranate extracts in diabetic rats on blood glucose, serum lipid, total cholesterol LDL and pancreatic lipid peroxidation (22, 23, 24, 25).

Hepatic harm is the most commonplace downside of most NSAIDs. Liver is the most important organ inside the body which maintain homeostasis of the internal body conditions (24, 26). Hepatoprotective marketers are those compounds, which mitigate the liver injury resulting from hepatotoxic retailers. The fundamental objectives to determine the phytochemicals in *Punica granatum* root extract and to test the hepatoprotective activity of *Punica granatum* in CCl4 triggered liver cells in rabbits with the aid of biochemical evaluation of ALT and AST of plant extract to govern the hepatitis disorder (27, 28).

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RESEARCH METHODOLOGY

Experimental Animal

Female domestic rabbits weighing 1.0-1.4kg were used for this study. These animals were kept in the animal house in wooden cages maintained at 12 h light and12 h dark. The animals were given a balanced rabbit's diet consisting of green leaves, fodder, pulses and water ad libitum. A measured amount of fresh food was replenished daily at 9.00am and 4.30pm.

Identification of Plant

The plant was identified from Department of Botany PMAS Arid Agriculture University Rawalpindi.

Preparation of Extract

The roots were dried and made into a coarse powder with the help of electric grinder. About 400gm of grinded plant material was subjected to Soxhlet extraction (650-750C) employing ethanol as solvent. The solvent was evaporated at 400 C to obtain the extract. The obtained extract was golden yellow in colour and was stored in refrigerator until use.

Phytochemical Screening

Phytochemical analysis of ethanolic extract of *Punica granatum* was done to check the chemicals present in it and for this qualitative analysis was done. Phytochemical analysis of tannins, saponins, flavonoids, anthraquinones and alkaloids were done according to the method (25) was used.

Induction of Liver Injury in Rabbits

Mixture of 1:1(v/v) mixture of CCl_4 in olive oil was prepared for the induction of liver injury and weight of rabbits were also measured. Initially different doses were given to induce hepatitis in rabbits. For this purpose, animals were divided into four groups. First group was given a dose of 1.25ml/kg body weight, second was given a dose of 1.0ml/kg body weight, third was given a dose of 0.75ml/kg body weight and the fourth was given a dose of 0.5ml/kg body weight. 70% rabbits died from first, second and third groups. But all rabbits belonging to fourth group were alive. So dose was given in the ratio of the 0.5ml/kg body weight and this dose was proved effective. The dosage of each animal was calculated, at a dose of 1.25 ml/kg body weight. The calculated amount of mixture was administrated i.p.in all groups for eight days except normal control.

CCl4 Induction

Mixture of CCl4 with olive oil in 1:1 ratio was injected intraperitoneally to the rabbits after 24 hours for 8 days. Rabbits were held properly before injecting.

Evaluation of Liver Damage

To evaluate the liver damage by CCl_4, one day after administration of 8th injection of CCl_4, the blood was collected from the thigh of all the rabbits. The blood samples were allowed to coagulating for 30 minutes at 37 ^{o}C. The clear serum was separated by centrifugation at 3000 rpm for 10 minutes. Sera were subjected to biochemical estimation of different parameters, such as asparate amino-transferase (AST) and alanine amino-transferase (ALT).

Treatment of Liver Injured Animals with the Extract of *Punica granatum*

Experimental Design

Rabbits were divided into four groups. Each group consists of six animals (n=6) as follow:

Normal Group: Normal and healthy rabbits.

Control Group: The liver damaged rabbits that received CCl_4 + olive oil i.p. for 7 days.

Treatment Group-I: Rabbits that received CCl_4 + olive oil for 7 days and then treated with extracts of *Punica granatum* at amount of 500mg orally for 10 days.

Treatment Group-II: Rabbits received CCl_4 the same as in Group-I, but extracts of *Punica granatum* at amount of 1000mg was given orally for 10 days.

Evaluation of Liver Function during Study

Biochemical Tests

The blood samples were collected and allowed to coagulating for 30 min. at 37 ^{o}C. The clear serums were separated at 3000 rpm for 10 min. and subjected to the estimation of biochemical

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analyses, asparate amino-transferase (AST) and alanine amino-transferase (ALT).

Estimation of the Activity of AST & ALT

1ml of substrate was pipette into two tubes A and B and placed in a water bath at 37°C for few min. To A, 0.2ml of serum was added and shaken gently to mix. One hour later in the case of AST, and after 30 min for ALT, 1ml dinitrophenyl hydrazine was added and 0.2ml sera to other (control) and allowed to stand for 20 min at room temperature. 10ml of 0.4N sodium hydroxide was added to all tubes, mixed well and was read at 520nm after 5 min in a colorimeter. For standard 1ml of working standard was taken and up to 1.2ml with water and proceeded as above. For standard blank, 1.2ml of water was taken and processed as above.

STATISTICAL ANALYSIS

The data obtained by the parameters was statistically evaluated by One Way ANOVA at 0.05. The mean value ±SEM were calculated for each parameter. The changes in biochemical parameters by the plant extracts in treated groups against the liver injured groups were analyzed. The mean differences within the treated groups were calculated by Least Significant Difference (LSD) Test.

RESULTS

PHYTOCHEMICAL ANALYSIS

The phytochemicals present in plant extract of *Punica granatum* was identified with different reagents. The results showed that alkaloids, saponins, sterols, steroids, terpenoids, flavonoids, tannins, phlobatannins and cardiac glycosides were present, while anthraquinones and cumarins were absent in the extract as shown in Table 1.

Table 1. Qualitative analysis of ethanolic extract of *Punica granatum* roots

Sr. No	Constituents	Observation	Presence /Absence
1	**Alkaloids**	Precipitation, Turbidity	+
2	**Saponins**	Formation of Emulsion	+
3	**Anthraquinones**	No reaction	**Nil**
4	**Coumarins**	No reaction	**Nil**
5	**Sterols**	Pink to purple color	+
6	**Steroids**	Green color	+
7	**Terpenoids**	Reddish brown color	+
8	**Flavonoids**	Yellow coloration	+
9	**Tannins**	Intense green and then black color	+
10	**Phlobatannins**	Red precipitation	+
11	**Cardiac glycosides**	Brown ring form	+

Measurement of ALT

ALT test was carried out to evaluate the functioning of liver of experimental animal. The result showed the values of normal, liver injured and control reported in table 2 and figure 1.

Table 2. Measurement of ALT in rabbits against hepatitis (N=6)

Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2) Mean value ±SEM

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Sr.No.		Days	N.G	C.G	T1	T2	ANOVA (P<0.05)	Remarks
1.	ALT	D_0	218±1	211±1	212±2	212±1	0.03	Significant
2.		D_8	215.6±1.52	149±1	93±1	88±1	0.03	Significant
3.		D_{16}	213.6±2.08	55±1	77±1	57±1	0.03	Significant

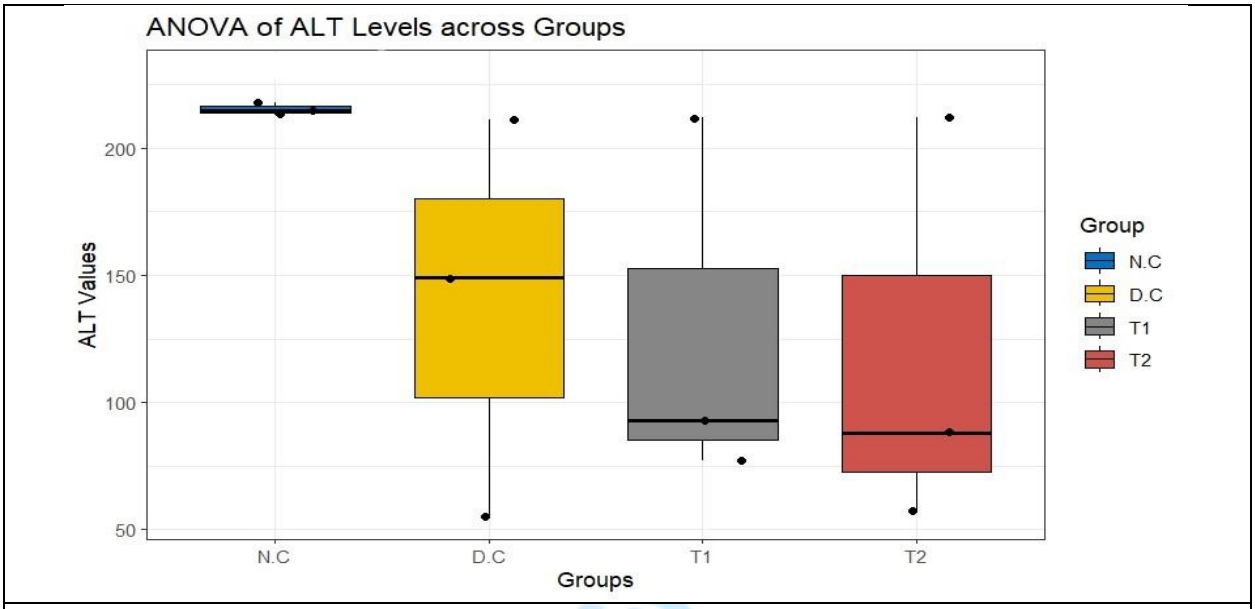

Figure 1(A): ANOVA Plot for measurement of ALT in rabbits against hepatitis
Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

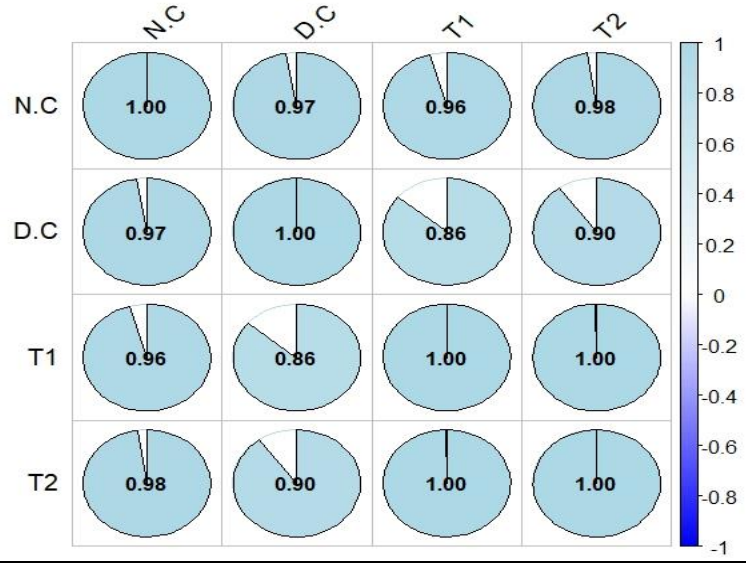

Figure 1(B): Heatmap Corrplot Plot for measurement of ALT in rabbits against hepatitis
Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

Furthermore, after one-way ANOVA test performed LSD test and Games-Howell post hoc test used to determine the relationship between the Dose Concentration of ALT in the plant extract with respect to number of days against the Hepatitis disease as shown in table No 3 and

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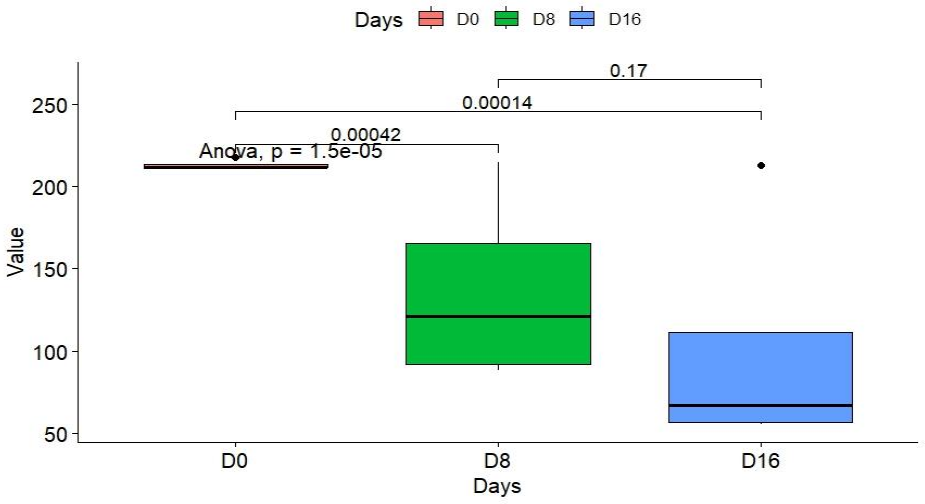
figure 2. The result indicated that the plant extract was useful to induced the liver cells in the rabbits to protect against the harmful diseases

Table 3: Mean difference of samples within the different treatment groups present of ALT in rabbits against hepatitis (n=6) for Least Significant Difference

Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

Mean difference of samples within the different treatment groups of ALT present in rabbits against hepatitis					
Variables	**N.G** $\bar{X}$ = 215	**C.G** $\bar{X}$ = 138	**T1** $\bar{X}$ = 127	**T2** $\bar{X}$ = 119	**REMARKS** LSD (P <0.05) After One Way ANOVA Test
N.G $\bar{X}$ = 215	~~~~	77	88	96	Significant
C.G $\bar{X}$ = 138		~~~~~	11	19	Significant
T1 $\bar{X}$ = 127			~~~~~~~	8	Non-Significant
T2 $\bar{X}$ = 119				~~~~~	**Result** Increased Dose concentration of root extract within the samples by ALT test effectively induced liver cells in rabbits
LSD Value	Critical value within samples group of ALT =14.8				

Figure 2: Comparison mean difference of samples within the different treatment groups ALT present in rabbits against hepatitis for Games-Howell Boxplot



Measurement of AST

AST test was carried out to evaluate the liver functioning of experimental animals. The result showed AST values of normal, liver injured, and control were reported in table 4 and figure 2.

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Table-4: Measurement of ALT in rabbits against hepatitis (N=6)
Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)
Mean value ±SEM

Sr.No.		Days	N.G	C.G	T1	T2	ANOVA (P<0.05)	Remarks
1.	AST	D_0	40±1	40.66±1.52	40±1	44.33±1.52	0.04	Significant
2.		D_8	41.66±1.52	200±1	133±1.52	145±1	0.04	Significant
3.		D_{16}	40.33±1.52	80±1	38±1	70±1	0.04	Significant

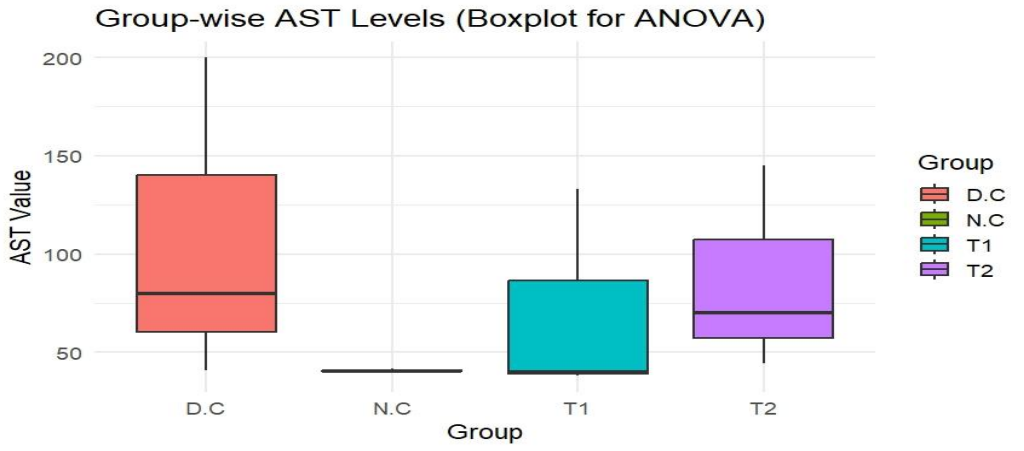

Figure 2(A): ANOVA Plot for measurement of AST in rabbits against hepatitis
Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

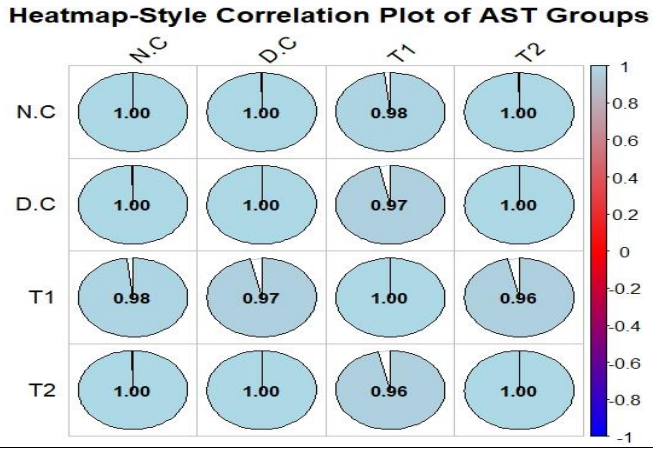

Figure 2(B): Heatmap Corrplot Plot for measurement of ALT in rabbits against hepatitis
Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

In the same way after one-way ANOVA test performed LSD test and Games-Howell post hoc test used to determine the relationship between the Dose Concentration of AST in the plant extract with respect to number of days against the Hepatitis disease as shown in table No 5 and figure 3. The result indicated that the plant extract was useful to induced the liver cells in the rabbits to protect against the harmful diseases

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Table 5: Mean difference of samples within the different treatment groups of AST present in rabbits against hepatitis (n=6) for Least Significant Difference

Normal group (N.G), Control group (C.G), Treated group 1 (T1), Treated group 2 (T2)

Mean difference of samples within the different treatment groups AST present in rabbits against hepatitis					
Variables	**N.G** $\bar{X}$ = 40	**C.G** $\bar{X}$ = 106	**T1** $\bar{X}$ = 70	**T2** $\bar{X}$ = 86	**REMARKS** **LSD (P <0.05)** **After One Way ANOVA Test**
N.G $\bar{X}$ = 40	-----	-66	-27	-45	Non-Significant
C.G $\bar{X}$ = 106		-------	36	20	Significant
T1 $\bar{X}$ = 70			--------	-16	Non-Significant
T2 $\bar{X}$ = 86				-------	**Result** **Increased Dose concentration of root extract within the samples by AST test effectively induced liver cells in rabbits**
LSD Value	The critical value of LSD = 12.4				

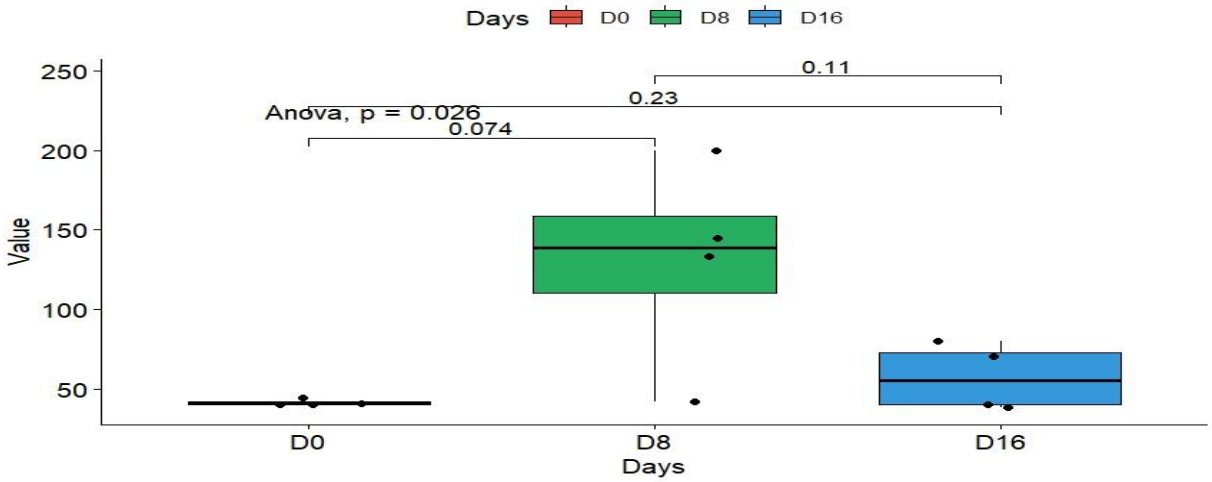

Figure 3: Comparison mean difference of samples within the different treatment groups of AST present in rabbits against hepatitis for Games-Howell Boxplot

DISCUSSION

The result indicated that the increased concentration of the root extract helpful to increase the activity of the liver cells to control the disease of injured livers in rabbits by biochemical analysis of ALT and AST (29). The result indicated that the root extract of *Punica granatum* was useful to induced the liver cells in the rabbits to protect against the harmful diseases like hepatitis as compared to its low concentration dose by Least Significant Difference (LSD) test. Similar results also reported by (26, 27) that pomegranate extract responsible to decreased the activities of colon cancer cells through the combined effect of antioxidant and anti-inflammatory cell signaling in rats. In the similar way, it was indicated that

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pomegranate extract may have used against the prostate cancer in human as chemoprotective agent (30, 31). Pomegranate extract have used to control the tumor-promoting effects in mouse skin and also responsible to decrease the harmful effect of ROS which can changes in DNA of cancer cells to form cells malignant, induced the proliferation of cancer cells, and activate the expression of tumor cells to form metastasis (32, 33).

It is a global problem and its harmful effects are increasing day by day. it is indicated that cancer act as a killer disease and its effect on any age group of people (17). In this age of industrialization exposure to toxins is widespread and entry of chemicals into our body produces many adverse effects in man and other beings. The liver is one of most important organs which damaged rigorously due to the persistent exposure of toxins it may be a drug, a pesticide, an industrial effluent or any other chemical entity (34,35). In the developing countries viral hepatitis is a common disease. However mostly for the treatment of hepatic disease there is no effective drug (36,37). To develop hepato-protective agent against hepatic malfunctioning is the need of hour. The development of hepato-protective drugs from plants is now become necessary due to cost; a considerable amount of research has been carried out in this regard (38, 39).

The use of different part of plants to treat the harmful diseases has been commonly used in various parts of the world for many years. Their motivation could be scientifically defensible by modern pharmacology and medicine. These plants may serve as new drugs source, especially by recent scientific knowledge (40). Many crude drugs have been developed in the past two decades, as a substitute medicine for the cure of various liver diseases. There is a beat of global understanding about the significance of many plants to protect the liver damaged by hepato-toxins.

CONCLUSIONS AND IMPLICATIONS

Punica granatum root extract have good source of phytochemical compounds such as phenols which are responsible for major pharmacological responses and used against the tumor cells.

AUTHOR CONTRIBUTIONS

Irfan Mumtaz, Usman Ahmad: Writing-original draft, Conceptualization, conducted experiment. **Irfan Mumtaz, Usman Ahmad:** Conceived the Idea, overall management of the work. **Muhammad Ali Raza:** Analysis and Interpretation of Results, Visualization. **Irfan Mumtaz:** Data Collection and field experiment layout preparation. **Muhammad Ali Raza and :** Reviewed the original draft. All the authors reviewed the results and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data and supplements available on request to the corresponding author.

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Institutional Review Board Statement

Ethics Review Committee of PMAS Arid Agriculture University Rawalpindi Pakistan.

Informed Consent Statement

Not Applicable

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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