Original Article

COMPARISON OF HEPATOPROTECTIVE ACTIVITY OF *EUGENIA JAMBOLANA* FRUIT WITH *PUNICA GRANATUM* FRUIT IN DIET INDUCED HYPERLIPIDEMIC RATS

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OBJECTIVE: To compare the hepatoprotective effects of *Eugenia jambolana* (EJ) fruit pulp with *Punica granatum*(PG) fruit in diet induced hyperlipidemic rats at the same dose level.

METHODS: This experimental randomized control study was conducted on seventy five male albino rats over a period of 14 weeks in University of Health Sciences Lahore. Rats were divided into five groups labelled A, B, C, D and E. There were fifteen rats in each group. Group A was kept as normal control, groups B, C, D and E were given hyperlipidemic diet for the whole duration of study i.e. fourteen weeks. In group B no further intervention was done, group C and D were given ethanolic extract of Eugenia jambolana and Punica granatum in same dose of 200mg/kg respectively for eight weeks. Group E was given combination of both in same dose of 200mg/kg each of both extracts for same duration. Serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were measured at zero and six weeks to confirm hyperlipidemia. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Creatinine phosphokinase (CPK) were measured at zero, six and fourteen weeks to observe any significant change in their levels as compared to normal and hyperlipidemic rats at week zero and six respectively.

RESULTS: At fourteen weeks, statistically no significant difference was found between groups A, C and E for serum ALT levels ($p>0.05$). For AST $p>0.05$ for group A vs E and C vs D only. CPK levels were not brought back to normal in any of the groups rather $p>0.05$ in B vs E. However it was significant ($p<0.05$) in all other groups for CPK.
Baseline (at zero weeks) serum lipid profile, ALT, AST and CPK levels showed no significant difference in means of all the groups with value of $p>0.05$ on intergroup comparison. At six weeks rats in groups B,C,D and E were found to be higher lipid profile ($p<0.05$) with deranged ALT and CPK levels as compared to control group A ($p<0.05$). However serum AST levels were not significantly deranged at this time ($p>0.05$). Fifteen rats of group A had significant lower levels of serum TC, HDL-C, LDL-C, TG, ALT and CPK when compared to 60 rats of groups B, C, D and E ($p<0.05$).

CONCLUSION: In male albino hyperlipidemic rats Eugenia jambolana fruit pulp extract alone and its combination with Punica granatum fruit pulp was equally effective in lowering serum ALT levels rather bringing them back to normal over a treatment period of eight weeks with continued hyperlipidemic diet. Serum AST levels were not deranged at six weeks but the levels remained normal with combination treatment only. However none of the treatment options could bring CPK levels back to normal rather one of the groups showed deranged CPK levels at the end of study.

KEYWORDS: ALT, AST, CPK, Rats, Punica granatum, Eugenia jambolana.

INTRODUCTION:

Natural products are the source of synthetic and traditional herbal medicine[1].The therapeutic efficacy of many indigenous plants has been described by traditional herbal medicinal practitioners for various ailments[2]. Consumption of plant foods is also associated with lower risk of CVD and hypertension[3]. Hyperlipidemia is one of the greatest risk factors contributing to prevalence and severity of cardiovascular diseases[4]. Baseline increase in serum liver enzymes is commonly seen in patients who are at high risk of coronary heart disease or are suffering from it. It is usually secondary to co morbid conditions, like obesity, dyslipidemias and diabetes, sharing properties of nonalcoholic fatty liver disease[5].
Almost half of the deaths in developed countries and one-fourth in the developing world are due to the diseases related to atherosclerosis with deranged lipid profile being its root cause[5]. Simvastatin (HMG Co A reductase Inhibitor) is world widely used antihyperlipidemic drug but is associated with increase in serum transaminase levels and raised CPK levels leading to myopathies in some cases[6]. Flavonoids extracted from gingko, soya bean, and some other plants have been reported as the antioxidants and could be beneficial as antihyperlipidemic agents[4].
The pomegranate, Punica granatum belongs to Punicaceae family. The therapeutic potential of this mystical fruit has been explored and is found to be useful in prevention of cancer, cardiovascular disease and diabetes. The extract of flower of Punica granatum has shown to decrease cardiac Triglycerides content along with reduction in plasma total cholesterol and triglycerides[7]. A few studies in the past have also shown improvement in liver function tests in animals with hepatotoxicity induced experimentally. The beneficial effect is due to antioxidant chemicals present in PG fruit[8].
Eugenia jambolana (EJ) is popularly known as Jamun in Pakistan and its seeds have been used for the treatment of diabetes and dyslipidemias[9]. In our previous study we conducted the experiment for comparison in antihyperlipidemic effects of flavonoids rich fruits EJ with world widely used antihyperlipidemic drug Simvastatin in experimental hyperlipidemic rats. Interestingly the fruit extract was found to be as effective as the HMG CoA reductase inhibitor simvastatin [10]. EJ is also a rich source of flavonoids possessing strong antioxidant and anti-inflammatory activity and has been shown to improve the liver function tests in experimental animals[9].
We designed this study to compare the liver protective effects of flavonoid rich fruit E.jambolana and Punica granatum in experimental hyperlipidemic rats. We also took this opportunity to observe their combined effect at the same dose level.

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MATERIAL AND METHODS:

This interventional study with control group was randomized by randomly generated computer numbers and was carried out after approval from the ethical committee of University of Health Sciences (UHS) Lahore in the animal house of UHS. Seventy five male albino rats were purchased from National Institute of Health (NIH), Islamabad. They were 4-5months old with weigh ranging from 180-220g. Five groups were made with 15 rats in each of them and were labeled as A,B,C,Dand E depending upon the type of intervention done in each of the groups. Periodic light and dark cycles were maintained throughout the study. For the first one week, all rats were fed on standard rat diet. Animals were weighed twice daily to calculate the dose of extracts to be administered.

EXPERIMENT:

All the animals of groups B,C,Dand E were fed on 2% cholesterol diet till the end of study, comprising duration of 14 weeks with water *ad-libitum.*

Group A was labeled as normal control and

was given regular rat diet till end of study.

Group B was hyperlipidemic control.

Group C (interventional group):
This group was given EJ fruit pulp extract in dose of 200mg/kg per oral, once daily, starting from six weeks for next eight weeks.

Group D (interventional group):
This group was given Punica granatum fruit extract 200mg/kg per oral, once daily starting from week six for the next eight weeks.

Group E (interventional group):
This group was given combination of EJ fruit pulp extract and PG fruit extract 200mg/kg per oral each and was started on week six and given for next eight weeks.
Fruit extract of EJ and PG was given orally as a single daily dose of 200mg/kg/day each.

Preparation of 2% cholesterol diet:
Cholesterol 2gm, extra pure manufactured by Scharlau (Spain) and cholic acid 500 mg, minimum 98% manufactured by Sigma-Aldrich (Germany) was mixed with 97.5 grams of rat diet and was given the form of pellets.

Preparation of Ethanolic Extract of Eugenia jambolana Fruit:
2kg of EJ was bought from local fruit shop in Lahore. It was certified by a qualified botanist of Hagler Bailley Pakistan (Pvt.) Ltd. using taxonomic rules. One liter of absolute Ethanol was procured from Merck (Germany) and the fruit pulp was separated from the seeds and was dipped in a stopped conical flask for 48 hours at room temperature with occasional stirring. Dark purple coloured solvent was formed in the flask which was filtered and then was put in a rotary vacuum evaporator (Heidolph, Laborota 4002) at 45^{o}C. Alcohol got evaporated and thick viscous extract was freeze dried using Labconco, Frezone 2.5 at -40^{o}C temperature under vacuum for 6 hours so that the moisture was completely removed. Hundred grams of extract was yielded from two kg fruit pulp. It was kept in a dark coloured air tight glass bottle in the refrigerator at 2 to 8^{o}C to be used throughout the study[13].

Preparation of ethanolic extract of Punica granatum fruit:
Punica granatum was bought from local fruit market of Lahore. It was also identified by botanist of Hagler Bailley Pakistan (Pvt.) Ltd. using taxonomic rules. Two kilogram grains of fruit were separated from the peel. They were air-dried from direct sunlight. The dried fruit was then grinded using electric grinder and was dipped in one liter of Absolute Ethanol, Merck (Germany) in a stopped conical flask for 48 hours. The mixture was kept at room temperature with stirring occasionally. The dark red solvent was formed in the flask which was filtered and then was put in a rotary vacuum evaporator (Heidolph, Laborota 4002) at 45^{o}C. Alcohol got evaporated and thick viscous extract was freeze dried using Labconco, frezone 2.5 at -40^{o}C temperature under vacuum for 6 hours so that the moisture was completely eliminated. Hundred grams of extract was obtained from two kg fruit pulp. It was kept in a dark coloured air tight glass bottle in the refrigerator at 2 to 8^{o}C to be used throughout the study[13].

Simvastatin:
Simvastatin was procured as simvastatin pure 98%-101% from Biocon limited, India.

COLLECTION OF BLOOD SAMPLE:

Cardiac puncture technique was utilized to collect blood sample of rats in vacutainers. Two rats from each group were sacrificed on day 1 of week one to obtain the baseline readings.
To confirm hyperlipidemia after six weeks of high cholesterol diet, three rats were randomly picked and 3ml of blood was collected by cardiac puncture to carry out liver function tests and CPK levels. Rats of group A served as normal control. On last day of week 14, all the remaining rats were sacrificed to observe the effects of drugs given for 8 weeks. Blood serum was obtained after centrifugation at 3000 rev/min for 15min[14]. Randox laboratory kits were used to measure serum ALT, AST and CPK levels using semi automatic clinical chemistry analyzer.

STATISTICAL ANALYSIS:

Results were expressed as mean ± S.E.M. The

difference between groups were assessed by ANOVA (analysis of variance) followed by Post hoc tukey test. Statistical significance was considered as $p<0.05$. SPSS version 20.0 was used to carry out statistical analysis.

RESULTS:

Table-1 shows the mean values of serum TC, HDL, LDL and TGat the start of experiment and after feeding the rats on hyperlipidemic food for six weeks. Table 2 shows results of alanine aminotransferase, aspartate aminotransferase and creatinine phosphokinase at 0, 6 and 14 weeks with extracts of EJ, PG and their combination.

Results in table-1 show that the difference in lipid profile parameters was not significant between groups A,B,C,D and E ($p>0.05$) at the start of experiment. Animals in group B,C,D and E who were fed on hyperlipidemic diet showed significantly higher lipid profile parameters in comparison to normal control group A with $p<0.05$ at six weeks.

Ethanolic extracts of fruit pulp of EJ (group C) and its combination with Punica granatum fruit (group E)in table 2 depicts significant reductions in serum ALT levels ($p<0.05$) at the end of fourteen weeks. Rather the deranged ALT levels at six weeks were brought back to normal after eight weeks of continued treatment with EJ alone (group C) and combination of EJ and PG fruit extract (group E). Group A vs C vs E showed vaule of $p>0.05$ at the end of fourteen weeks. Serum AST levels were however not deranged due to diet induced hyperlipidemia at six weeks but statistically significant difference was observed at fourteen weeks with continued hyperlipidemic diet along with combination of EJ and PG extract. Serum CPK levels remained deranged till the end of study in group E ($p>0.05$) only. The remaining groups showed statistically significant improvement.

Table-1: Lipid profile parameters at zero and six weeks

Groups	TC (mg/dl) Zero weeks	six weeks	TG (mg/dl) zero weeks	six weeks	HDL(mg/dl) zero weeks	six weeks	LDL (mg/dl) zero weeks	six weeks
A	58.50±1.9	60.00±4.06	93.30±3.10	87.26±2.03	19.90±1.3	21.23±1.4	19.94±4.5	21.31±3.06
B	57.45±1.4	183.00±4.99	94.80±5.60	188.66±5.36	21.50±2.0	16.43±2.1	16.99±4.5	128.83±4.8
C	68.60+3.2	188.56±9.99	90.00+1.60	194.76±11.1	27.15+2.9	12.76±1.2	23.45±6.4	136.8±7.78
D	61.30±2.30	193.50±4.53	93.25±5.75	202.00±4.93	23.40±3.80	16.40±0.51	19.25±0.35	136.70±4.15
E	68.20±6.2	183.23±13.80	106.50±3.50	188.63±11.31	25.00±1.00	17.86±1.79	21.90±5.9	127.63 ± 13.23
p-value	0.187	0.000	0.196	0.000	0.365	0.035	0.892	0.000

*All values are expressed as Mean ±S.E.M

TC=Total cholesterol, TG=Triglycerides, HDL=High density lipoprotein, LDL=Low density lipoprotein

Table-2: ALT, AST and CPK levels at zero, six and fourteen weeks

Groups	ALT (U/L)			AST (U/L)			CPK (U/L)		
weeks	zero	six	fourteen	zero	six	fourteen	zero	six	fourteen
A	52.80±1.6	54.3±0.7	53.9±1.4	67.20±2.0	90.2±0.9	62.0±0.8	110.95±9.4	122.6±6.6	152.0±1.6
B	57.85±2.55	76.7±3.0	84.3±2.6	62.10±0.60	95.5±3.2	92.2±2.1	178.25±23.25	220.0±14.5	339.6±6.1
C	60.80±1.6	80.5±0.6	58.5±1.2	59.40±0.6	97.5±8.0	72.0±1.2	142.20±5.8	265.3±35.5	293.7±6.4
D	56.95±3.2	87.2±3.8	65.6±2.6	61.65±1.8	97.8±1.9	71.7±1.7	152.75±46.7	292.6±12.2	409.8±10.8
E	62.0±1.0	86.0±1.5	57.6±1.5	64.50±1.7	100.3±6.4	63.9±1.7	137.75±11.5	308.0±6.8	337.6±7.6
P-Value	0.144	0.000	0.000	0.080	0.704	0.000	0.487	0.000	0.000

*All values are expressed as Mean ±S.E.M

ALT= Alannine aminotransferase, AST=Aspartate aminotransferase and CPK=Creatinine phosphokinase

DISCUSSION:

Literature review elucidates the facts that phytotherapy is safe and less toxic[14]. So, for the management of the diseases which require lifelong therapy fruit extracts can be a good option as in most Asian countries where the use of folk medicine is prevalent, the search for traditional cures is a common practice and is well accepted[15].

The antihyperlipidemic activity of Eugenia jambolana has been studied and was found to be as effective as Simvastatins (HMGCoAreductase inhibitors) in experimental rats[10]. Simvastatin is however notorious for raising serum transaminase and CPK levels leading to myopathies in some cases. Our study is unique because a comparison is made between the hepatoprotective activity of Eugenia jambolana and Punica granatum fruit extracts along with their combination and serum CPK levels were also measured. The purpose was to compare their effects at the same dose level. In our study the rats were divided into five groups and to make them hyperlipidemic four groups i.e. B,C,Dand E were given 2% cholesterol diet and one group i.e. group A was kept on normal rat food to serve as normal control. The rats fed on high cholesterol diet were found to have deranged ALT and CPK levels. Continued hyperlipidemic diet till the end of the study further deteriorated liver enzymes in group B when compared at six and fourteen weeks showing that hyperlipidemia can derange liver function tests. The results of this experimental study show that ethanolic extracts of fruit pulp of EJ and its combination with PG fruit cause significant reductions in serum ALT levels. Rather the deranged ALT levels at six weeks were brought back to normal after eight weeks of continued treatment with EJ alone (group C) and combination of EJ and PG fruit extract (group E). Group A vs C vs E showed vaule of $p>0.05$ at the end of fourteen weeks. Serum AST levels were however not deranged due to diet induced hyperlipidemia but statistically significant difference was observed at fourteen weeks after continued hyperlipidemic diet till the end of study.

The results of our study were consistent with study conducted in 2014 by Shaban NZ who demonstrated improvement in liver function tests by PG juice[16]. In a study conducted by Moneim AA in 2011 pomegranate peel extract in same dose of 200mg/kg has been shown to cause a significant reduction ($p < 0.05$) in alkaline phosphatase and bilirubin levels. The plant possesses significant antioxidant activity[8]. Another carried out by Hafiz TA in 2016 on murine malaria induced hepatic injury in mice proved improvement in liver function tests after treatment with PG peel extract. Significant reduction of the hepatic oxidative markers, glutathione, nitric oxide and malondialdehyde was also observed in these mice[17].

Eugenia jambolana has also been shown to reduce inflammation and swelling of liver and spleen and has been used traditionally for many years for treatment of liver dysfunctions. The chemical constituents of EJ contain gallic acid, mallic acid, ellagic acid and a large number of other antioxidant flavonoids[18].

Results of serum CPK were intriguing, in one of the groups serum CPK levels remained deranged till the end of the study while statistically significant improvement was observed in all other groups but none of the options brought serum CPK levels back to normal. This finding suggests that the fruit extract of E.Jambolana and Punica granatum may be having different mechanism of action which needs to be explored further. We assume that antioxidant chemicals of E.Jambolana and Punica granatum cause reduction in liver enzyme.

Flavonoids inhibit Hydroxy methyl glutrylreductase (key enzyme involved in cholesterol biosynthesis) also it activates the enzyme 7 a-hydroxylase which accelerates cholesterol metabolism[19].

The aim was to evaluate which of the two fruit extracts has a better effect on liver function tests and CPK levels at the same dose level. We also had an opportunity to study the effect of combination therapy of Eugenia jambolana and Punica granatum fruit extracts to observe if there is an increase or decrease in response. As shown by our results the effect of EJ pulp extract alone was

as good as the combination therapy over reducing serum ALT levels which were brought back to normal.

For future studies we can perform a bioassay guided isolation of bioactive flavonoids from both fruit extracts as done by other researchers[20]. Their structures can be elucidated and hepatoprotective evaluation can be performed.

CONCLUSION:

Medicinal plants have low cost, they are less toxic and free from side effects therefore, can be started in early years of life to prevent the development of hyperlipidemias and associated comorbidities like raised liver function tests. Combination of ethanolic extracts of Eugenia jambolana and Punica granatum fruit pulps was as effective as Eugenia jambolana fruit extract alone in lowering serum ALT levels. In future, these plants can be used as antihyperlipidemic agents.

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3	Dr. Maryam Rashid	Helped in drafting & finalize the manuscript
4	Waqas Latif	Helped in analyzing the data
5	Saadia Noreen	Helped in perfuming lab analysis