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Glycemic Response of Citrus Limon, Pomegranate and Their Combinations in Alloxan-Induced Diabetic Rats

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Abstract: Present study was performed to investigate the effect of Citrus limon and Pomegranate juices alone and their combinations on blood glucose and plasma insulin levels in alloxan induced diabetic rats as compared to diabetic control. Decrease in blood glucose level by Citrus limon was highly significant in dose dependant manner but there was highly significant increase in plasma insulin levels at moderate dose and significant increase at high dose. However Pomegranate showed highly significant decrease in blood glucose, in inverse dose dependant manner i.e. maximum effect was observed at low dose. Whereas there was highly significant increase in plasma insulin level at all three doses. Whereas combination dose group CPJ-1 (0.4+5ml/kg) showed highly significant response on blood glucose and plasma insulin, which was maximum effect obtained among all treated groups. These results suggest that Citrus limon and Pomegranate has phytochemical and other essential nutrients which have reduced blood glucose levels, that works synergistically in combination. So it may be concluded that the response of citrus limon and pomegranate on blood glucose may be due to presence of flavonoids in these juices and a synergistic effect is observed by their combination.

Key words: Blood glucose, Insulin, Pomegranate juice, Citrus limon juice.

INTRODUCTION

Diabetes is the condition of different etiologies, like insulin insufficiency, disturbance in metabolism of carbohydrates, proteins, fats and oxidative stress. Uncontrolled diabetes may leads to cardiovascular disease, stroke and even death (Onaolapo *et al*., 2011). According to a report of World Health Organization 2006,diabetes has been persistently affecting persons, and it is one of the leading causes of 80% deaths in developing countries. Annual data of 1990-2010 showed that number of diabetic patients, almost became tripled than data of 1980-1990 (CDC, 2012). Many synthetic hypoglycemic agents are currently available but are either too expensive or have undesirable side effects on chronic use (Consumer report, 2012). In traditional system of medicine role of plant based dietary products have been continuously increasing as alternative treatment due to minimal side effects and reduce costs. Till today many plants have been evaluated for their hypoglycemic effects. Since plants and plant derived phytochemical, have great potential to treat and control diabetes (Behera,Yadev, 2013). Most of the research was carried out to evaluate the anti-diabetic effect of these phytochemical and suggests antioxidant action (Mollace *et al*., 2011; Yin *et al*., 2011).Fruits and vegetables areroutinely consumed as diet and are easy way to reach these phytochemicals. Fruits rich in these phytochemical are pomegranate, orange, grapefruit and citrus limon etc.

Flavonoids found in Citrus limon ([L.] Burm.f.) includes hesperidin, quercitrin, eriocitrin, didymin and naringin (Nijveldt *et al*., 2001). Role of naringin have been reported for its antidiabetic effects (Pari *et al*., 2010). It is well known that vitamin C, folate, carotenoid and phenolic contents abundant in Citrus limon are beneficial to human health. Hence Citrus limonshows antioxidant, anti-inflammatory, gastro protective, antifungal and anti-microbial effects (Dhanavade *et al*., 2011; Chun *et al*., 2008; Viuda *et al.,* 2008; Rozza *et al*., 2011).

Phytochemical found in Pomegranate (Punicagranatum, Punicaceae) includes ellagitannins, gallotannins and anthocyanins (Fischer *et al*., 2011).Role of pomegranate in diabetes have been reported by Betanzos-Cabrera *et al*., 2011 due to its flavonoids. Pomegranate has its role in many other diseases like acidosis, dysentery, diarrhea, helminthiasis, hypercholestremia, hemorrhage and cancer (Anoosh*et al*., 2010; Tehranifar*et al*., 2010).

Phytochemicals may be extracted from fruit juices (Deyhim *et al*., 2006; Xu.,*2008*), but duration of extraction and storage conditions may affect bioavailability of these phytochemicals. Hence to avoid this loss we used freshly squeezed juices of Citrus limon and Pomegranate fruits.

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The objective of this study was to evaluate the blood glucose lowering effect of Citrus limon and Pomegranate juice alone as well as in combinations and also to measure serum insulin level in alloxan-induced diabetic rats.

MATERIAL AND METHOD

Animals:

Study was carried out after approval of the proposal by board of advance studies and research, University of Karachi on adult male Wister rats with mean body weight of 220±10 grams. Animals were kept under controlled condition of temperature 23±2°C and humidity 50-60%, with free access to food and water. Five rats were housed in each plastic cage measuring 32"x18"x16".

Citrus Limon Juice:

Citrus limon was purchased from local market and identified by center of plant conservation, University of Karachi. The voucher specimen no C.L 11-11 was deposited in department of Pharmacognosy, University of Karachi. Citrus limon were squeezed by hand to yield fresh juice which was then filtered. Citrus limon juice was administered in three doses according to body weight i.e. 0.2ml/kg, 0.4ml/kg and 0.6ml/kg through oral route and was considered as low (LCLD), moderate (MCLD), and high Citrus limon dose (HCLD) respectively.

Pomegranate Juice:

The Pomegranate was also purchased from local market, identified by center of plant conservation, University of Karachi. The voucher specimen no P.G 11-12 was deposited in department of Pharmacognosy, University of Karachi. The fruits were peeled, squeezed, filtered and fresh juice was yielded. Juice was given through oral route in three doses according to body weight i.e. 2ml/kg, 5ml/kg and 8ml/kg and was considered as low (LPD), moderate (MPD) and high dose (HPD) respectively.

Combinations of Citrus Limon and Pomegranate:

Citrus limon and Pomegranate juice were given orally in two combination doses i.e. 0.4ml/kg Citrus limon+5ml/kg Pomegranate juice and 0.2ml/kg Citrus limon+8ml/kg Pomegranate juice and was abbreviated as CPJ-1 (0.4+5ml/kg) and CPJ-2 (0.2+8ml/kg). All doses were given once daily through oral route.

Drug and Induction of Diabetes:

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of alloxan monohydrate (Sigma chemicals, USA) 180mg/kg body weight, in normal saline. The glucose level in plasma was determined at 72h. After the administration of alloxan (Rohilla *et al*., 2012).Rats with blood glucose concentration more than 250mg/kg were considered diabeticand used for the study.

Design of Experiment:

All rats were divided into eleven groups each comprising of ten animals. Control group received saline, while all other groups induced diabetes were given Citrus limon (three groups), pomegranate(three groups), while two groups received Citrus limon and Pomegranate in combination, one group received standard drug glibenclamide and one group served as diabetic control (received saline). All doses were given by gastric intubation on once daily basis for the period of six weeks. Entire study was performed under NCCL guideline (Wayne, 1998). At the end of the experimental period, the animal were deprived of food overnight and sacrificed by decapitation and blood samples were collected in gel tubes and 3.2% sodium citrate containing tubes (9:1v/v).

Estimation of Blood Glucose:

Blood glucose was determined by two methods: 1: Accu-Chek glucometer by taking blood from tail vein of rats, after 72h of intraperitoneal injection of alloxan to check the induction of diabetes. 2: Blood collected in gel tubes, serum was separated by Humax 14K centrifuge at 2000rpm for 10min and then commercial kit of Glucose (Human Diagnostic, Germany) was used to estimate blood glucose byHumaLyzer3000 Human Germany.

Estimation of Plasma Insulin:

Blood was collected in 3.2% sodium citrate tubes. Plasma was separated by Humax 14K centrifuge at 3000rpm for 15 minutes and then insulin was estimated using commercial ELISA insulin kit (Accu-Bind, Elisa Microwells, U.S.A).

Statistical Analysis:

Data entry and analysis was performed using Superior Performance Statistical Software (SPSS) version 20. Data was presented as mean ± SD with 95% confidence interval. ANOVA followed by post hoc was performed

for comparisons of values with control. Values of $p<0.05$ were considered statistically significant and $p<0.005$ highly significant.

RESULTS AND DISCUSSION

Results:

Table I shows the effect of Citrus limon on blood glucose level and plasma insulin in diabetic rats and control animals. There was highly significant decrease in blood glucose at LCLD, MCLD and HCL Dindose dependant manner, in comparison to diabetic controls. While highly significant increase in insulin level at MCLD and significant increase at HCLD. However there was no change in plasma insulin level at LCLD.

Table I: Effect of Citrus limon on blood glucose level and plasma insulin.

Parameters	Groups				
	Control	Diabetic control	LCLD	MCLD	HCLD
Blood Glucose (mg/dl)	83.8±8.98	354.8± 29.21	210.0± 31.43**	197.6± 11.03**	190.0± 10.46**
Plasma Insulin (µU/ml)	12.3±0.90	7.04± 0.36	7.0± 0.53	10.3± 0.78**	9.0± 0.84*

n=10, Values are mean ± S.E.M
*P ≤ 0.05 significant as compared to diabetic control
**P ≤ 0.005 highly significant as compared to diabetic control
LCLD: Low Citrus limon dose 0.2ml/kg/day;
MCLD: Moderate Citrus limon dose 0.4ml/kg/day;
HCLD: High Citrus limon dose 0.6ml/kg/day;

Table II shows the effect of Pomegranate on blood glucose level and plasma insulin in diabetic treated rats and control animals. There was highly significant decrease in blood glucose at LPD and MPD, while HPD showed significant decrease in blood glucose in comparison to diabetic controls. While highly significant increase at LPD, MPD and HPD in dose dependant manner was observed, in comparison to diabetic controls.

Table II: Effect of Pomegranate on blood glucose level and plasma insulin.

Parameters	Groups				
	Control	Diabetic control	LPD	MPD	HPD
Blood Glucose (mg/dl)	83.8± 8.98	354.8± 29.21	203.0± 24.25**	222.6± 13.22**	273.6± 39.16*
Plasma Insulin (µU/ml)	12.3± 0.90	7.04± 0.36	10.1± 0.67**	10.9± 0.67**	11.0± 0.62**

n=10, Values are mean ± S.E.M
*P ≤ 0.05 significant as compared to diabetic control
**P ≤ 0.005 highly significant as compared to diabetic control
LPD: Low Pomegranate dose 0.2ml/kg/day ;
MPD: Moderate Pomegranate dose 0.4ml/kg/day;
HPD: High Pomegranate dose 0.6ml/kg/day;

Table III shows the effect of combination doses of Citrus limon and Pomegranate with standard drug on blood glucose level and plasma insulin in diabetic and control animals. There was highly significant decrease in blood glucose at CPJ-1 and Glibenclamide treated animals, while significant decrease was observed at CPJ-2 in comparison to diabetic controls. There was highly significant increase in plasma insulin at CPJ-1, CPJ-2 and Glibenclamide treated animals in comparison to diabetic control.

Table III: Effect of combination doses of Citrus limon and Pomegranate on blood glucose level and plasma insulin.

Parameters	Groups				
	Control	Diabetic control	CPJ-1	CPJ-2	Glibenclamide
Blood Glucose (mg/dl)	83.8± 8.98	354.8± 29.21	177.5± 7.42**	281.5± 36.99*	165.8± 9.43**
Plasma Insulin (µU/ml)	12.3± 0.90	7.04± 0.36	11.5± 0.64**	10.60± 0.71**	11.7± 0.65**

n=10, Values are mean ± S.E.M
*P ≤ 0.05 significantly different as compared to diabetic control
**P ≤ 0.005 highly significant as compared to diabetic control
CPJ-1: combination dose 0.4+5ml/kg/day; i.e. 0.4ml citrus juice and 5ml pomegranate juice
CPJ-2: combination dose 0.2+8ml/kg/day ; i.e. 0.2ml citrus juice and 8ml pomegranate juice

Discussion:

Diabetes mellitus has been considered as one of the major health problem, all around the world. Health promoting effect of fruits and vegetables has been widely studied for the prevention or treatment of chronic diseases. The suggested mechanism and beneficial effects by these phytochemicals present in fruit and vegetables is due to their anti-oxidation properties (Sunagawa *et al*., 2004), since stress due to oxidation and

freeradicals generation is considered as major cause of hyperglycemia (Kawahito *et al.,* 2009) as well as in alloxan-induced diabetes in experimental rats (Rohilla *et al.,* 2012).

Phytochemicalsare abundantly found in fruits and act as strong anti-oxidants. Pomegranate and Citrus limon are richin these phytochemicals (Violi *et al*., 2010). However little work has been done to determine the effect of Citrus limon and Pomegranate juices on blood glucose and plasma insulin level.

Result of the present study showed highly significant decrease in blood glucose level by Citrus limon in dose dependant manner, suggesting the need to work on higher dose, to obtain desired blood glucose lowering effects. This effect might be due to presence of naringin. Since naringin is reported to produce hypoglycemic effect due to its strong anti-oxidation property (Pari *et al*., 2010). Whereas Citrus limon showed highly significant increase in plasma insulin levels at moderate dose. This effect may be due to the presence of vitamin C in Citrus limon which acts as strong antioxidant (Violi *et al*., 2010).Results of present study are in consistency with the study of Antunes *et al*., (1998), who showed that vitamin C is less effective at high dose then low dose. In present study plasma insulin level was significantly increased at high dose and no change was observed at low dose. One possible explanation could be that, dosage adjustment is necessary to maintain drug concentrations within their therapeutic windows (Tripoli *et al*., 2007). It is suggested that different duration and dosage of Citrus limon juice may play important role in all of their observed effects.

Present study revealed inverse dose dependant decrease in blood glucose bypomegranate i.e. low dose showed maximum effect than moderate and high dose. While highly significant increase in plasma insulin were observed. On the basis of these results it could be postulated that pomegranate induced hypoglycemic effect is not only due to effect on plasma insulin, but may also be due to some other mechanisms involved at molecular level. Since polyphenols found in pomegranate juice has the ability to enhance activity and expression of paraoxnase 1 (PON 1), aglycoprotein enzyme expressed in liver and excreted in blood (Betanzos-Cabrera *et al*., 2011). It has strong anti-oxidant effect and lowers the risk of atherosclerosis (Fischer *et al*., 2011).

There was highly significantly decrease in glucose level at CPJ-1 inpresent study which was maximal response among all treated groups in the study. This might be due to synergic effect of naringin present in Citrus limon and polyphenols in Pomegranate. Moreover naringin and vitamin C has been reported to produce hypoglycemic and hypocholesterolemic effects due to their strong anti-oxidant properties (Pari *et al*., 2010). While polyphenols and vitamin C in Pomegranate may show these beneficial effects due to their strong antioxidant property (Tezcan *et al.,* 2009; Tehranifar *et al*., 2010).

There was highly significantly decrease in glucose level in CPJ-1 group which was maximal response among all treated groups in the study. This might be due to presence of naringin Citrus limon and polyphenols in pomegranate as antioxidant. All antioxidants may produce a synergistic effect which may provide strengthening to the B-cells of pancreas to release more insulin. Moreover vitamin C present in Pomegranate may be responsible for beneficial effects together with vitamin C in Citrus limon (Ajith*et al*., 2009; Tezcan *et al*., 2009; Tehranifar *et al*. 2010).

Conclusion:

From the results of present study it may be concluded that combination of Citrus limon and Pomegranate is most effective in moderate doses, however further studies on more combination doses of Citrus limon and Pomegranate are required to reveal the role of these substances to reduce blood glucose levels and combat diabetes mellitus.

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