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RESEARCH ARTICLE

Polyherbal Formulation Prevents Hyperglycemia by Modulating the Biochemical Parameters and Upregulating the Insulin Signaling Cascade in Alloxan Induced Hyperglycemic Rats

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ABSTRACT

Diabetes mellitus is a metabolic disorder having serious consequences on health and is becoming the 3rd most fatal disease worldwide. The current study was designed to prepare and explore the antihyperglycemic potential of a polyherbal formulation comprising aqueous extracts of *Momordica charantia*, *Syzygium cumini*, *Acacia nilotica*, *Elettaria cardamomum*, *Cicer arietinum* L, *Foeniculum vulgare* and *Gymnema sylvestre*. Hyperglycemia was induced by alloxan monohydrate (150 mg/kg). After induction of diabetes, polyherbal formulation was administered in graded doses 200, 400 and 600mg/kg to treated groups I, II and III respectively. Polyherbal formulation was found to be rich in phytoconstituents on phytochemical analysis. Antihyperglycemic potential of polyherbal formulation was determined through biochemical and gene expression analysis. Results of the study revealed that polyherbal formulation significantly reversed the alloxan monohydrate induced hyperglycemia in rat models by improving the biochemical parameters in dose dependent manner. Highest dose of polyherbal formulation (600 mg/kg) significantly reduced the serum glucose (142.60±3.12 mg/dl), glycosylated hemoglobin (6.62±0.27%) and increased the serum insulin (16.87±1.53 U/L) levels in comparison to diabetic control group having serum glucose (375.20±8.98 mg/dl), glycosylated hemoglobin (13.92±0.70%) and insulin (6.26±1.13 U/L) levels respectively. Moreover, polyherbal formulation enhanced the performance of pancreatic β cells by upregulating the expression of PDX-1, INS-1 and INS-2 genes (insulin signaling cascade). Conclusively, the results of current study indicated the potent hypoglycemic properties of polyherbal formulation.

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INTRODUCTION

Diabetes mellitus/hyperglycemia is a metabolic disorder resulting from defects in insulin secretion and/or insulin action on receptor site and is mainly characterized by elevated blood glucose levels. Both hereditary and environmental factors are involved in the pathogenesis of diabetes mellitus. It is becoming the 3rd most fatal disease worldwide. There are mainly two types of diabetes mellitus, including type 1 (insulin dependent) and type 2 (non-insulin dependent). In case of type 1 diabetes mellitus body does not produce enough insulin while type 2 diabetes mellitus is characterized by development of resistance for insulin action and this insulin dependence is temporary (Pan *et al*., 2015).

The epidemiological data has revealed that currently 246 million people are suffering from diabetes mellitus world widely and this figure is estimated to be increased up to 300 million by the year 2030 (Bhaskar *et al*., 2016). Numerous pathogenic aspects are supposed to be involved in the progression of diabetes mellitus. These may include age, obesity, genetics, ethnicity, reduced physical movement, immune disorders, flaws in pancreatic beta cells and insulin resistance on receptor site (Leach, 2007). Type 1 diabetes mellitus is mainly caused by genetic disturbances and autoimmune destruction of pancreatic beta cells (Gillespie, 2006). Type 2 diabetes is mainly caused by increased glucose production and glucagon secretion along with peripheral insulin resistance in body (Kahn *et al*., 2006). Gestational diabetes is another type of diabetes which is mainly associated with pregnancy and caused by

inability of pancreatic beta cells as well as development of insulin resistance (Siddiqui *et al*., 2013). Persistent hyperglycemic conditions may lead towards macro and microvascular complications (Siddiqui *et al*., 2013).

For the treatment of type 2 diabetes mellitus different drug therapies are used. Sulfonylureas are mainly used to treat type 2 diabetes mellitus but severe side effects (hypoglycemia, weight gain) are associated with these marketed drugs (Krentz and Bailey, 2005). So, there is a quest to develop new antidiabetic drugs with increased effectiveness and minimum side effects. Medicinal plants are easily available and used for the treatment of various diseases (Abbas *et al*., 2017a, 2017b, 2017c; Hussain *et al*., 2017; Idris *et al*., 2017; Zaman *et al*., 2017) but the main problem associated with these herbal therapies is lack of standardization (Salimifar *et al*., 2013). So, there is a need for scientific validation of herbal therapies to evaluate their safety and efficacy as well as the side effects associated with these herbal remedies. The concept of polyherbalism is becoming more common for the treatment of diabetes mellitus to produce synergistic effects of herbs which can increase the efficacy and decrease the toxicity of single herbs. Literature has shown that polyherbal formulations have more therapeutic efficacy as compared to single herbs (Rajendran *et al*., 2014).

Momordica charantia (bitter gourd), *Syzygium cumini* (Jamun). *Acacia nilotica (*Kikar), *Elettaria cardamomum* (cardamom), *Cicer arietinum L* (chickpea), *Foeniculum vulgare* (fennel) and *Gymnema sylvestre* (Ghurmar boti) are the plants which are used for the treatment of various diseases in traditional medicine system. Antidiabetic potential of individual plant is well known (Paul *et al*., 2015) but the synergistic effects are not clear. Keeping in view the previous literature, current project was designed to scientifically validate the polyherbal formulation prepared from the above-mentioned plants and to evaluate the antihyperglycemic potential of this formulation. Moreover, the effects of polyherbal formulation on proliferation and regeneration of pancreatic beta cells were also determined by measuring the expression level of different genes including PDX-1, INS-1 and INS-2 involved in Insulin signaling cascade.

MATERIALS AND METHODS

Plant material: In the present study, equal ratio of aqueous extracts of seeds of *S. cumini, E. cardamomum, C. arietinum*, *F. vulgare*, fruits of *M. charantia*, *A. nilotica* and leaves of *G. sylvestre* were used for the preparation of polyherbal formulation. The plant materials were identified by a taxonomist, from Department of Botany, University of Agriculture Faisalabad, Pakistan where a voucher specimen for each plant has been deposited (voucher specimen nos: (*S. cumini*: 21138), (*E. cardamomum*: 21139), (*C. arietinum*: 21140), (*F. vulgare*: 21141), (*M. charantia*: 21142), (*A. nilotica*: 21143), (*G. sylvestre*: 21144)). The plant materials were shade dried and crushed into powder form by using mechanical grinder. After passing through mesh sieve (No.120) separately, 100g of dry powder of each plant was soaked in 250ml of water separately for two hours and boiled for 30 minutes. The collected extracts were then filtered and dried. The powders of each extract were mixed in equal ratio to form the polyherbal formulation.

Phytochemical analysis: The following phytochemicals were tested for their presence in polyherbal formulation by using the standard methods with slight modifications. Flavonoids ($FeCl_3$ test) (Raman, 2006), tannins ($FeCl_3$ test), alkaloids (Mayer's reagent test) (Evans, 1997), phenols ($FeCl_3$ test) (Mace, 1963), saponins (Foam test) (Shabi *et al*., 2014), proteins (Biuret test) (Brain and Turner, 1975), fixed oils and fats (Stain test) (Shabi *et al*., 2014), glycosides (Auwal *et al*., 2014) and steroids (Auwal *et al*., 2014).

Experimental design and induction of experimental diabetes: Ninety healthy young albino rats weighing 180-200g were categorized into 6 groups, each group comprised of 15 rats. Group I assigned as Control, Group 2 as Diabetic control on Alloxan monohydrate, Group 3 as Positive control on glibenclamide, Group 4 as Treated I (Polyherbal formulation 200 mg/kg), Group 5 as Treated II (Polyherbal formulation 400 mg/kg), Group 6 as Treated III (Polyherbal formulation 600 mg/kg). Prior the initiation of study an approval was obtained from the Graduate Studies Research Board, University of Agriculture, Faisalabad, Pakistan. Experimental diabetes was induced by single intraperitoneal injection of alloxan monohydrate (150 mg/kg) in groups 2, 3, 4, 5 and 6. Criteria of diabetic rats for inclusion in the investigation was the blood glucose concentration greater than 300mg/dl (Aruna *et al*., 1999). After induction of diabetes, glibenclamide and graded doses of polyherbal formulation were administered to groups 3, 4, 5 and 6 respectively for 8 weeks through intragastric tube. At the end of study, rats were slaughtered by cervical dislocation and blood samples were collected to separate serum for further analysis. The pancreatic sections of rats were snap frozen in liquid nitrogen for gene expression analysis.

Biochemical analysis

Estimation of serum glucose, insulin, glycosylated hemoglobin, leptin and liver glycogen activity: Serum Glucose, Insulin, Glycosylated hemoglobin and Leptin levels were measured through corresponding commercially available diagnostic kits. Liver glycogen activity was determined through method given by Plummer (1971).

Determination of liver function markers: Serum ALT, AST, ALP and bilirubin levels were measured by using corresponding commercially available diagnostic kits.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR): RNA isolation was performed by using Trizol reagent (ThermoFisher, USA) (Liu and Patel, 1995) and subjected to cDNA synthesis with equal RNA concentration in each sample, according to manufacturer's instructions using the RevertAid cDNA synthesis kit (ThermoFisher Scientific). qRT-PCR was performed by using Maxima SYBR Green/ROX Master Mix (ThermoFisher Scientific). Expression of PDX-1, INS-1 and INS-2 genes was analyzed. Primer sequences for all genes are given in Table 1. The cDNA was denatured for 15 seconds at 95°C for all genes. Then primers were annealed at 52°C for 25 seconds, and the extension time was 20 seconds at 72°C for 40 cycles. All these steps were

accompanied by 95°C denaturation for 10 minutes at the start of a single cycle. Expression levels of these genes were normalized to β-actin. The 2x(-ΔΔct) method was used to analyze RT-PCR data.

Data analysis: The data was expressed as mean±SE. Statistically data were evaluated by applying one-way analysis of variance (ANOVA). For statistical differences among means Duncan multiple range (DMR) test was applied. P≤0.05 was considered as the significant difference among groups.

RESULTS

Phytochemical analysis of polyherbal formulation: Aqueous extract of polyherbal formulation was subjected to qualitative phytochemical analysis. Results indicated the presence of phenols, flavonoids, alkaloids, fixed oils, saponins, tannins, glycosides, proteins and terpenoids, while steroids were not present in this formulation (Table 2).

Biochemical analysis

Effect of polyherbal formulation on serum glucose, insulin, glycosylated hemoglobin, leptin and liver glycogen concentrations: Alloxan monohydrate significantly (P≤0.01) increased the serum glucose and glycosylated hemoglobin levels in groups 2, 3, 4, 5 and 6. Standard antidiabetic drug (glibenclamide) and polyherbal formulation in treated groups I, II and III significantly (P≤0.01) reduced the serum glucose and glycosylated hemoglobin levels after 8 weeks of study. However, serum insulin and leptin levels were significantly reduced in diabetic rats. Glibenclamide significantly increased (P≤0.01) the serum insulin and leptin levels. Oral administration of polyherbal formulation also increased (P≤0.01) the serum insulin and leptin levels in dose dependent manner. Moreover, liver glycogen concentration was significantly decreased (P≤0.01) in diabetic rats. Glibenclamide and polyherbal formulation in treated groups I, II and III significantly increased (P≤0.01) the liver glycogen concentration (Table 3).

Effect of polyherbal formulation on liver function markers: The levels of ALT, AST, ALP and bilirubin were significantly raised (P≤0.01) after induction of diabetes in all groups compared to control group. The increased levels of ALT, AST, ALP and bilirubin were reduced in glibenclamide and polyherbal formulation treated groups I, II and III (200, 400 and 600mg/kg). Polyherbal formulation reduced the levels of hepatic enzymes in dose dependent manner (Table 4).

qRT-PCR analysis for PDX-1, INS-1 and INS-2 genes: The mRNA expression level of PDX-1, INS-1 and INS-2 genes was assessed in control, diabetic control and polyherbal formulation treated III (600 mg/kg) groups. As biochemical analysis revealed the maximum effectiveness of highest dose of polyherbal formulation, so in gene expression analysis only highest dose of polyherbal formulation was compared with control and diabetic control groups. The expression level of above mentioned genes in the control group was considered as reference to calculate and normalize the expression in the other groups. Comparing with control rats, the mRNA expression level of PDX-1, INS-1 and INS-2 genes in diabetic control group rats was suppressed. Polyherbal formulation (600mg/kg) treatment significantly increased (P≤0.01) the PDX-1, INS-1 and INS-2 genes expression (Fig. 1).

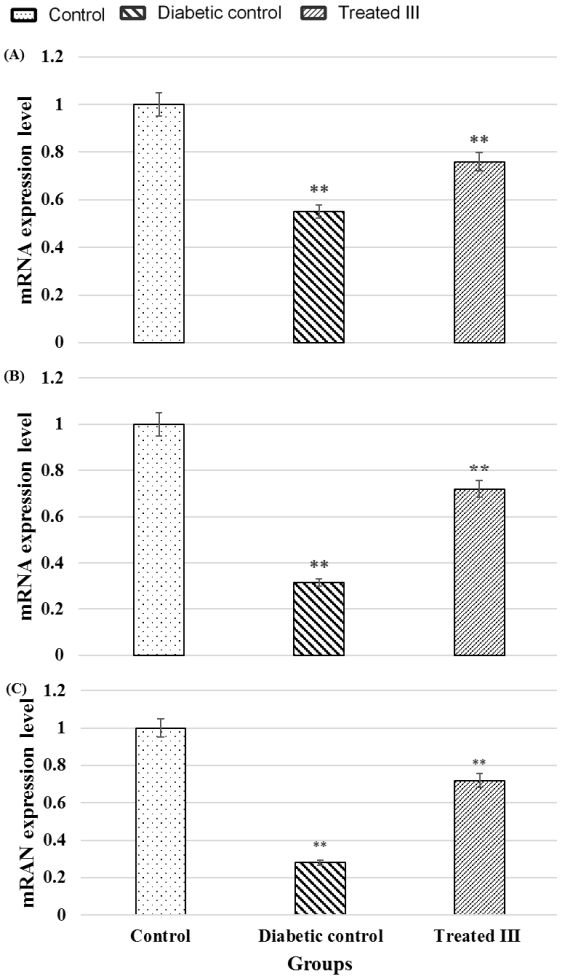

Fig. 1: Expression profiles of (A) PDX-1 gene in rat pancreata of control, diabetic control and polyherbal formulation treated III (600mg/kg) groups (n=3). (B) INS-1 gene in rat pancreata of control, diabetic control and polyherbal formulation treated III (600mg/kg) groups (n=3). (C) INS-2 gene in rat pancreata of control, diabetic control and polyherbal formulation treated III (600 mg/kg) groups (n=3). (**) Values are significantly different (P≤0.01).

Table 1: Oligonucleotides (Primers) used for amplification of Beta actin, PDX-1, INS-1 and INS-2 genes

INS-1F	AGGCTCTGTACCTGGTGTGTG
INS-1R	CGGGTCCTCCACTTCACGAC
INS-2F	GGAGCGTGGATTCTTCTACACA
INS-2R	AGTGCCAAGGTCTGAAGGTCAC
PDX-1 F	TCCCGAATGGAACCGAGACT
PDX-1 R	TTCATCCACGGGAAAGGGAG
Beta actin F	CGAGTACAACCTTCTTGCAGC
Beta actin R	TATCGTCATCCATGGCGAACTG

Table 2: Phytochemical analysis of polyherbal formulation

Phytochemicals	Present/Absent
Phenols	+
Flavonoids	+
Alkaloids	+
Fixed oils	+
Saponins	+
Tannins	+
Glycosides	+
Proteins	+
Steroids	-
Terpenoids	+

Present (+), Absent (-).

Table 3: Effect of polyherbal formulation on serum glucose, insulin, glycosylated hemoglobin, leptin and liver glycogen levels in alloxanized diabetic rats

Parameters	Control	Diabetic control	Positive control	Treated I (200 mg/kg)	Treated II (400 mg/kg)	Treated III (600 mg/kg)
Serum glucose (mg/dl)	114.20±3.97e	375.20±8.98a	128.40±3.14d	188.00±2.59b	175.20±2.76c	142.60±3.12cd
Serum Insulin (U/L)	17.96±1.67a	6.26±1.13d	17.54±2.10b	12.69±1.46c	14.66±1.43bc	16.87±1.53b
Glycosylated hemoglobin (%)	5.72±0.12e	13.92±0.70a	6.05±0.45d	8.92±0.99b	8.05±0.61bc	6.62±0.27d
Leptin (ng/ml)	4.08±0.15a	1.78±0.21c	3.08±0.21b	2.43±0.16bc	2.68±0.18b	2.78±0.17b
Liver glycogen (mg/g)	45.76±2.81a	9.24±2.88d	39.24±2.89b	23.24±2.96c	29.52±2.91bc	33.72±1.88b

One-way ANOVA followed by DMR test. Values are expressed as mean±SE with different letters within a row differ significantly from each other (P≤0.01).

Table 4: Effect of polyherbal formulation on liver function markers in alloxanized diabetic rats

Parameters	Control	Diabetic control	Positive control	Treated I (200 mg/kg)	Treated II (400 mg/kg)	Treated III (600 mg/kg)
Serum ALT (U/L)	36.05±3.56d	88.07±4.96a	41.96±3.52c	61.33±4.35b	55.43±3.64b	47.41±3.48bc
Serum AST (U/L)	42.03±1.80d	131.60±3.56a	45.10±2.79c	73.20±3.14b	67.82±3.14bc	49.49±2.05c
Serum ALP(U/L)	110.60±3.47d	193.80±6.76a	124.40±3.66c	151.60±2.86b	142.80±3.07bc	132.00±2.77bc
Serum bilirubin (mg/dl)	0.45±0.15c	1.83±0.27a	0.56±0.19b	1.19±0.35ab	0.99±0.28ab	0.69±0.20b

One-way ANOVA followed by DMR test. Values are expressed as mean±SE with different letters within a row differ significantly from each other (P≤0.01).

DISCUSSION

Plenty of plants and herbs are presently used for the treatment of diabetes mellitus. According to WHO reports, approximately 80% of population is depending upon traditional therapies particularly in Asian countries, for the treatment of diabetes mellitus and other primary health care problems. Herbal products are used alone or in combination with conventional therapies. Although herbal therapies possess good safety profile in comparison to allopathic medicines but unfortunately scientific validation for safety and efficacy of herbal remedies is missing (Udoamaka and Jose, 2014). In present investigation antihyperglycemic activity of polyherbal formulation was evaluated through phytochemical, biochemical and gene expression analysis using rat models.

The polyherbal formulation tested in the present study contained aqueous extract of *Momordica charantia, Syzygium cumini, Acacia nilotica, Elettaria cardamomum, Cicer arietinum L, Foeniculum vulgare* and *Gymnema sylvestre*. Phytochemical analysis of formulation under study revealed the presence of phenols, flavonoids, alkaloids, triterpenes, saponins, proteins and tannins. However, steroids could not be detected in the formulation. Triterpenes create a huge structurally varied group of natural complexes that possess numerous biological properties. Biologically, flavonoids are utmost recognized for their free radical scavenging properties (Usunobun *et al*., 2015). Several research studies have shown that these compounds possess numerous antihyperglycemic mechanisms. They prevent the insulin resistance and regulate plasma/serum glucose and insulin levels (Nazaruk and Borzym-Kluczyk, 2014).

In this work, alloxan-induced diabetic rats were characterized by a significant increase in their serum glucose level, along with a significant decrease in their serum insulin level in comparison with the control rats as revealed in numerous previous research studies (Cheng *et al*., 2013). We found noteworthy decrease in blood glucose concentration in polyherbal formulation treated diabetic rats. Other researchers likewise indicated decrease in hyperglycemic conditions with medicinal plants based therapies (Ezeigbo *et al*., 2016). This decrease in glycemia could be associated either with enhancement in the insulin level because of positive impact of flavonoids present in formulation on the β-cells of pancreas or improvement in the transport of glucose to the peripheral tissues (Cheng *et al*., 2013). Further antidiabetic activity of the experimental polyherbal formulation was confirmed by significant increase in level of serum insulin, suggesting an insulinogenic potential of the formulation. The increase in serum insulin level directs that polyherbal formulation stimulated secretion of insulin from the remaining intact or regenerated pancreatic beta cells. In this background, several other medicinal plants have also been testified to exert antihyperglycemic potential through stimulatory effect on the release of insulin (Gireesh *et al*., 2009).

Data of the current study also revealed that polyherbal formulation significantly reduced the level of HbA1c in alloxan-induced-diabetic rats. This might be due to the fact that polyherbal formulation increases the utilization of glucose by tissues via impeding gluconeogenesis in liver and by stimulating the pancreatic β-cells due to the presence of terpenoids and other phytoconstituents for their insulin-like activity (Moses *et al*., 2016). In the current study, serum leptin level was significantly reduced in diabetic rats which might be associated with insulin deficiency. However, treatment of diabetic rats with glibenclamide and polyherbal formulation significantly raised the serum leptin level. Increased leptin levels contribute in the improvement of hepatic and skeletal insulin sensitivity (Yildiz and Haznedaroglu, 2006).

Liver glycogen content was estimated at the end of study. It was obvious from results that hepatic glycogen content reduced significantly after induction of diabetes. Reduction in glycolytic enzyme activities might be responsible for depletion of glycogen content in diabetic rats. Furthermore, glycogen deposition is impaired in diabetic rats which is directly associated with deficiency of insulin. However, administration of polyherbal formulation significantly improved the liver glycogen content in comparison to diabetic control group. The results of present study are consistent with recordings of previous research studies (Jayaprasad *et al*., 2016).

Metabolic disorders such as diabetes can cause damage of hepatocytes. The injury to hepatic cells is responsible for the release of intracellular elements into systemic circulation. Measurement of serum

concentrations of hepatic enzymes provides a valuable mean for scientific diagnosis of hepatic damage. Induction of diabetes mellitus with alloxan directed a noteworthy increase in the activity of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the diabetic rats in comparison to the control group. The Upsurge in the levels of hepatic enzymes in alloxan induced diabetes might be due to outflow of enzymes from the hepatic tissue into the plasma. The polyherbal formulation reduced the activity of ALT, AST and ALP. The reduction in hepatic enzyme levels was dose dependent. The results of current study are in agreement with previous research studies (Aja *et al*., 2015; Eguavoen *et al*., 2016). The findings of current study also showed that the experimental induction of diabetes markedly raised the level of serum bilirubin. The increment in serum bilirubin may occur because of the reduction of liver uptake, increase of bilirubin formation or conjugation. However, glibenclamide and polyherbal formulation treatment induced a massive decrease in serum bilirubin level (Naseer and Muhammad, 2014).

To explain the protective effects of polyherbal formulation, regeneration-related pathway such as insulin signaling pathway, was explored in the present study. To study the insulin signaling cascade expression level of genes including PDX-1, INS-1 and INS-2 was investigated in control, diabetic control and polyherbal formulation (600 mg/kg) treated animal models. From the results, it was obvious that alloxan monohydrate significantly ($P \leq 0.01$) decreased the mRNA expression level of PDX-1, INS-1 and INS-2 genes. The mechanism involved might be the ROS production by alloxan in pancreatic beta cells, leads towards reduced expression level of PDX-1, which in turn is responsible for the suppression of other genes involved in insulin signaling cascade, such as insulin 1 (INS-1) and insulin 2 (INS-2) (Siddique and Rabbi, 2016). Results of current study have shown that mRNA expression of PDX-1, INS-1 and INS-2 genes was reversed by the polyherbal formulation extract treatment.

Conclusions: Current study has revealed the antihyperglycemic potential of polyherbal formulation. Polyherbal formulation significantly reduced the alloxan monohydrate induced hyperglycemia and improved the biochemical parameters including serum glucose, insulin, leptin, liver glycogen content, ALT, AST, ALP and bilirubin. Polyherbal formulation also improved the performance of pancreatic endocrine tissues via upregulation of insulin signaling cascade. The current investigation also supported the presence of various phytoconstituents in polyherbal formulation which might be responsible for its antihyperglycemic potential by reducing the beta cells apoptosis.

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Authors contribution: This manuscript is based on Ph.D thesis of first author. WM and TK contributed to design the whole experiment. WM and BA performed biochemical and gene expression analysis. JAK participated in statistical analysis of data. All authors involved in discussing the contents of the manuscript and agreed to publication.

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