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Renal Protective Effect of Saffron Aqueous Extract in Streptozotocin Induced Diabetic Rats

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ABSTRACT

Background: Multiple-organ failure is the main cause of death in diabetes mellitus and hyperglycaemia induced oxidative stress is responsible for major diabetic complications including nephropathy. Medicinal plants with antioxidant activity may modulate oxidative status and improve the functions of multiple organs that affected by chronic hyperglycaemia in diabetes. Aim: The aim of the present study was to investigate on the beneficial effects of saffron aqueous extract (SAE) in streptozotocin (STZ)-induced diabetic rats by measuring the oxidative indicators, functional enzymes and histopathological studies in the kidney tissues. Methods and Material: The fasted rats were injected intraperitoneally (IP) by a single dose of a STZ (60 mg/kg body weight). After 72 hours, the animals with fasting blood glucose over than 250 mg/dl were considered diabetic and were used in the experimental groups; control (1), control drug (2), diabetes (3) and diabetes drug (4). The treatment was started on the 7th day after STZ administration with an IP injection of SAE (200 mg/kg body weight), six doses and weekly to groups (2 and 4). At the end of the experiment, fasting blood glucose, oxidative status and functional enzymes in kidney tissues were measured. Furthermore, histopathological studies were performed. **Results:** The results showed that SAE significantly decreased blood glucose and prevented weight loss in treated diabetic rats compared to untreated diabetic rats. Moreover, SAE improved oxidative balance and functional enzymes activities in the kidney tissues of Group 4 in comparison to Group 3. Also, histopathological studies showed the less injury in the kidney tissues of treated diabetic rats. Conclusion: Administration of SAE showed significant ameliorative effects on the biochemical, oxidative, and histopathological parameters on kidney tissues of treated diabetic rats.

Keywords: Antioxidant, Saffron aqueous extract, Kidney, Diabetes mellitus, Rat

INTRODUCTION

Diabetes mellitus (DM) is a group of longstanding disorders manifested by hyperglycaemia. Actiology of diabetes is related to defects in insulin secretion and/or insulin action that leading to hyperglycaemia [1].

It has been revealed that chronic hyperglycaemia, the hallmark of DM, leads to protein glycation and formation of advanced glycation products (AGE), which contribute to both microvascular and macrovascular complications. These complications affect the entire body specially, the eyes, nerves, kidneys, livers, blood vessels, heart and contribute to morbidity and mortality of diabetes [2-8].

In addition, hyperglycaemia can lead to high production of reactive oxygen species (ROS) and simultaneous reduction of the antioxidant defence mechanisms, which can cause oxidative stress. Therefore, hyperglycaemia induced oxidative stress due to the cellular function disruption and cellular damage has a crucial role in the development and progression of diabetic complications [9-11].

Diabetic nephropathy as a microvascular complication is responsible for about 30-40% of all end-stage renal disease cases [12]. When hyperglycaemia is maintained for a long time, nephropathy occurs due to the multiple cellular

mechanisms including, activation of protein kinase C (PKC) pathway, cytokines production, enhanced polyol pathway, increased formation of advanced glycation end products (AGE), increased oxidative stress and hexosamine pathway [9,13].

Due to the worldwide prevalence of diabetes, it seems essential to minimize the complications of this devastating disease. Common treatments for diabetes such as using of hypoglycaemic drugs and insulin administration may have undesirable side effects. In addition, most of treatments have not reported to prevent diabetes complications such as nephropathy [14]. Therefore, the using of medicinal herbs for diabetes treatment has received considerable attention because of suitable efficacy and fewer side effects than chemical agents. Saffron (Crocus sativus) is one of the medicinal plants that known as a red gold and belonged to Iridaceae family. Saffron commonly is consumed as a food flavouring and colouring, but it has many potential therapeutic effects to improve diseases such as depression [15], Alzheimer [16], inflammation [17] and cancer [18]. It has mentioned that crocin and crocetin as major carotenoids of saffron are responsible for antioxidant activity of its [19,20]. As well as saffron extract and its active ingredient, crocin have recognized to inhibit renal Ischemia Reperfusion (IR) in rats [21]. It is proposed that diabetes makes alterations in the activity of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase that leading to imbalance in oxidative status and damage of tissues [22]. On the other hand, saffron has antioxidant effects and there is a close dependence between the antioxidant effect of plants and anti-diabetic activity [23]. There are some reports that proposed antidiabetic effects of saffron [24-27], but possible association between SAE and kidney enzymes alterations of diabetic rats is not reported yet. Therefore, the present study aims to determine the effect of SAE on kidney biochemical and antioxidant enzymes (AST, ALT, ALP, GGT, LDH, SOD, GPX, CAT), lipid peroxidation and renal histopathological alterations in streptozotocin (STZ) induced diabetic rats.

MATERIALS AND METHODS

Preparation of saffron aqueous extract (SAE)

Powdered saffron (dried stigmas) was soaked in distilled water and was shaken in darkness and cool place for 3 days. The mixture was filtered by Whatman paper. After centrifugation, the supernatant was freeze dried and subsequently stored in freezer. For administration to rats, after weighting was dissolved in distilled water for injection.

Animal study

Thirty-two male Wistar rats weighing 200-220 g, 8 weeks old, were used in this study. Animals were housed under standard laboratory conditions (five rats in per cage, ambient temperature of $23 \pm 2^{\circ}$ C, under a 12-h light/12-h dark cycle and access to food and water ad libitum) and weighed weekly. All experiments performed in accordance with the health guidelines for laboratory animals that prepared by Shiraz University. After acclimatization period, the animals were accidentally divided to 4 groups as following: Group 1, healthy control rats or C group (received vehicle, n=7), Group 2, healthy rats that received SAE and determined as control drug or CD group (n=7), Group 3, diabetic control rats or D group (received STZ, n=9) Groups 4, diabetic rats that received SAE and determined as diabetic drug or DD group (n=9).

Animal ethics

The experiment was performed under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the protection of animals used for experimental purposes were considered.

Induction of diabetes and treatment with SAE

At first, streptozotocin (STZ, Sigma, USA) was dissolved in 0.1 M of sodium citrate buffer (pH=4.5) was kept on ice and immediately used. In order to access the appropriate dose, each rat was weighed a day before injection. Diabetes was induced by single intraperitoneal injection of STZ (60 mg/kg) to fasted rats of groups 3 and 4. After injection, the rats in two mentioned groups received 5 M solution of glucose for 12 hours to prevent of blood glucose reducing. About 72 hours later, the levels of fasting blood glucose were measured by using one touch glucometer. Then the rats with blood glucose levels higher than 250 mg/dl were selected as diabetic rats. One week later of STZ injection, the treatment of rats in 2 and 4 groups was begun by intraperitoneal injection of SAE (200 mg/kg), weekly, for 5 weeks. After the end of the experiment, fasting blood glucose of each group was measured. Then, the animals were killed under anaesthesia and kidney tissues in all groups were removed immediately. Each of kidney tissues was divided into two slices, half of the slices were fixed in 10% buffered formalin for histopathological studies and the other slice was frozen in liquid nitrogen and stored at -70° C for biochemical investigations.

Kidney homogenate preparation

To prepare the homogenized kidney tissue, 100 mg of wet kidney tissue was placed into 1 cc of cool phosphate buffer 100 mM (pH=7.4). The homogenization was performed by sonication device and centrifuged (750 g, for 15 min at 4° C). The soluble fraction was used for biochemical assessments.

Biochemical assays

Functional enzymes activities

At the time of experiment, the soluble fraction from above section was used for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ -glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities. All assays were performed by using commercial kits (Pars Azmoon, Tehran, Iran) and spectrophotometric methods.

Measurement of oxidative stress status

Superoxide Dismutase (SOD) activity

The supernatant was used for assessment of SOD activity by using a commercial kit (Randox, UK). The method of kit employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Glutathione peroxidase (GPx) activity

The Randox kit (UK) was used for measurement of GPx activity. This method is based on GPx activity for oxidation of Glutathione (GSH) by Cumene hydroperoxide. Then, in the presence of Glutathione Reductase (GR) and NADPH, the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+.

The decrease in absorbance at 340 nm is measured and the activity was determined based on the mentioned method that presented in the kit.

Catalase (CAT) activity

The catalase (CAT) activity in the kidney homogenate was assayed with hydrogen peroxide as the substrate by using a method that was based on the direct measurement of H_2O_2 decomposition [28].

Malondialdehyde (MDA) determination

Lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (535 nm) product, proportional to the MDA present in kidney homogenate [29].

Total protein measurement

Total protein of the homogenate kidney tissues was estimated by Lowry method and specific activity of mentioned enzymes were reported.

Histopathological examination

A portion of kidney tissue was fixed in buffered formalin (10%) and embedded in paraffin wax. Around 5 μ m-thick sections were made and stained with haematoxylin and eosin (H and E). The histopathological sections were examined under a light microscope.

Statistical analysis

All data were analyzed by using the SPSS 16.0 statistical package and expressed as mean \pm standard deviation.

Statistical evaluation of significant difference between the means of groups was performed with one-way analysis of variance (ANOVA), followed by Tukey test and p<0.05 was regarded as significant.

RESULTS

Effect of SAE on mortality

All animals in healthy groups (Group 1 and Group 2) were lived up to the end of the experiment. Mortality was 22.22% (2 of 9) and 11.11% (1 of 9) in untreated diabetic rats (Group 3) and diabetic rats received SAE (Group 4), respectively. Thus, SAE significantly decreased mortality (p<0.05).

Effect of SAE on body weight

The mean weight of rats at the period of the experiment is shown in Table 1. Results indicated that increase in body weight of treated rats with SAE (Group 2 and 4) compared to control groups (Group 1 and 3) was not statistically significant (p>0.05). But decrease in the body weight of diabetic groups was statistically significant when compared to control groups.

 Table 1 The mean of body weight in all groups at the period of treatment with SAE. The groups were healthy control rats

 (C), healthy rats that received SAE (CD), diabetic control rats (D) and diabetic rats that received SAE (DD)

Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5			
С	210.83 ± 10.04	215.2 ± 17.59	225.5 ± 17.84	240.67 ± 19.7	255.33 ± 13.99	262.67 ± 7.26			
CD	208 ± 17.31	218.17±10.8	229.25 ± 15.39	246.1 ± 10.56	260.2 ± 13.13	270.67 ± 13.12			
D	217.17 ± 6.01	203.83 ± 10.65	200.17 ± 16.12	200 ± 14.77	200.1 ± 14.44	196.17 ± 16.51			
DD	217.86 ± 4.41	205 ± 8.29	206.43 ± 8.77	211.29 ± 17.62	217 ± 16.62	217 ± 16.68			
The zero week in the table indicates the body weight at the first of the experiment (before of STZ injection);									
Values are the mean \pm SD									

Effect of SAE on the levels of fasting blood glucose

After the induction of diabetes and before treatment with SAE, fasting blood glucose significantly increased in diabetic rats (Group 3 and Group 4) compared to control groups (Group 1 and Group 2). As shown in Figure 1, SAE significantly decreased blood glucose levels in treated diabetic rats (Group 4) at the end of the experiment compared with untreated diabetic animals (Group 3) (p<0.05). While, the diabetic control rats (Group 3) exhibited significant hyperglycaemia during of the experimental period (p<0.05).



Figure 1 The mean of fasting blood glucose before and after treatment with SAE in all groups. The groups were healthy control rats (C), healthy rats that received SAE (CD), diabetic control rats (D) and diabetic rats that received SAE (DD). Values are the mean \pm SD. The different letters indicate the significance of the data that compares with another (P<0.05)

Effect of SAE on the activity of kidney functional enzymes

As shown in Table 2, there was a significant increase in the activity of ALT, AST, ALP and LDH in untreated diabetic group (Group 3) as compared to control groups (Group 1 and Group 2). While the activity of GGT enzyme showed a significant decrease in rats of Group 3 in comparison with control groups (Group 1 and Group 2). The results showed

that SAE can normalize the activity of mentioned enzymes. So that, about of ALT, AST, ALP and LDH activities there was not any significant difference between treated diabetic rats with SAE and control group (Group 1).

 Table 2 The effect of SAE on the specific activity of functional enzymes in kidney tissues. The activities were described as mean ± SD

Parameter	Group 1	Group 2	Group 3	Group 4			
ALT (U/gr)	$25.16\pm1.87^{\text{a}}$	$20.2\pm4.26^{\rm a}$	$44.75 \pm 12.72^{\rm b}$	$32.66 \pm 12.2^{\text{a,b}}$			
AST (U/gr)	12 ± 3.1^{a}	$10.5\pm3.7^{\rm a}$	$17.8\pm7.1^{\rm b}$	$15.2 \pm 7.2^{a,b}$			
ALP (U/gr)	124.01 ± 37.98^{a}	$148.41 \pm 49.42^{a,b}$	$201.88 \pm 46.025^{\rm b}$	$155.64 \pm 32.01^{a,b}$			
GGT (U/gr)	750.032 ± 106.44^{a}	$569.13 \pm 105.87^{a,c}$	$178.833 \pm 43.68^{\rm b}$	$453.33 \pm 130.01^{\circ}$			
LDH (U/gr)	$1422.5 \pm 223.94^{a,c}$	$1183.83 \pm 115.19^{\circ}$	$1985 \pm 369.74^{\rm b}$	$1627.167 \pm 297.49^{a,b}$			
Means with different superscript letters are significant at $P < 0.05$. A uses harmonic mean sample size = 7 rats							

Effect of SAE on GPx activity in kidney tissues

As shown in Figure 2a, the specific activity of GPx showed a non-significant decrease in untreated diabetic rats compared with control group (Group 1). But the administration of SAE significantly increased its activity in treated diabetic group (Group 4) in comparison with all groups (Group 1, Group 2 and Group 3) (p<0.05).

Effect of SAE on SOD activity in kidney tissues

Changes in SOD activity in all experimental groups are shown in Figure 2b. A significant decrease was observed in the activity of its in untreated diabetic rats versus respective control groups (Group 1 and Group 2) (p<0.05). Treatment of diabetic animals with saffron could significantly increase SOD activity compared with all groups (Group 1, Group 2 and Group 3) (p<0.05).

Effect of SAE on CAT activity in kidney tissues

Our results (Figure 2c) showed that there was no significant difference in CAT activity between untreated diabetic rats and control groups (Group 1 and Group 2). But SAE significantly increased its activity in treated diabetic rats (Group 4) compared with other groups (p<0.05).



Figure 2 The effect of SAE on the oxidative status in kidney tissues in all groups. (a): GPx activity, (b): SOD activity, (c): CAT activity, (d): MDA levels, the groups of the study were healthy control rats (C), healthy rats that received SAE (CD), diabetic control rats (D) and diabetic rats that received SAE (DD). Values are the mean ± SD. The different letters indicate the significance of the data that compares with another (P<0.05)

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Effect of SAE on the level of MDA in kidney tissues

As shown in Figure 2d, MDA levels as an index for lipid peroxidation or oxidative stress, significantly (p<0.05) increased in untreated diabetic rats in comparison with the control groups (Group 1 and Group 2). Our results indicated that the treatment of diabetic animals with SAE could significantly decrease MDA levels compared with untreated diabetic animals (p<0.05). So that, there was no significant differences in MDA levels between treated diabetic rats with SAE and control groups (Group 1 and Group 2).

Histopathological results

Kidney histological structure was normal in healthy groups (Group 1 and Group 2) (Figure 3a). Histopathological examination of the tissue sections showed significant increase of the glomerular volume in untreated diabetic rats in comparison with the control group. Most of kidney sections in untreated diabetic group showed interstitial nephritis with infiltration of lymphocytes and clear vacuoles in the renal tubule epithelium (Figures 3b, 3c and 3d). Diabetic rats treated with SAE revealed mild infiltration of lymphocytes in the interstitium and decrease of cytoplasmic vacuoles in comparison to untreated diabetic rats (Figures 3e and 3f).



Figure 3 Renal sections. a. Tissue section of a healthy mouse. b. Tissue sections of diabetic rats show an increase of the glomerular volume. c. There is interstitial nephritis with infiltration of lymphocytes (arrow) in tissue section of diabetic rats. d. Cytoplasmic vacuoles (arrows) are seen in diabetic rats. e. Treated diabetic rats show mild infiltration of lymphocytes in the interstitium (arrows). f. Decrease of cytoplasmic vacuoles is observed in treated diabetic rats (arrow). H&E, ×720

DISCUSSION

In the present study, the renal protective effect of SAE on the streptozotocin induced diabetic rats was performed for the first time. Our results indicated 72 h after the injection of STZ to animals, significant hyperglycaemia was observed in all rats (Group 3 and Group 4) (Figure 1). It has been proved that STZ (as a glucosamine-nitroso-urea compound) can develop destruction in pancreatic beta cells and thereupon hyperglycaemia due to the production of free radicals. As well as, the mean weight of untreated diabetic rats (Table 1) was less than the weight of healthy groups (Group 1 and Group 2). Decrease in the weight of diabetic rats is may be due to the loss of muscle (proteolysis) and adipose (lipolysis) tissues that dependent to insulin for using of glucose as fuel.

Long term hyperglycaemia has been proposed as a main contributor to begin and develop the microvascular complications of diabetes, including nephropathy by accumulation of advanced glycation end products (AGE) [30], activation of diacylglycerol (DAC) - protein kinase C (PKC) pathway [31], and over production of damaging free radicals [32]. As well as, hyperglycaemia has been reported as a main cause for glomerular hyper-filtration that results in nephropathy and tissue injuries in diabetes [33]. So, hypoglycaemic ability is an essential property to recognize an effective antidiabetic drug. Our data revealed that SAE administration to diabetic rats during the experimental period could increase body weight along with decrease in fasting blood glucose compared with untreated diabetic rats. These results are consistent with previous studies [24-27]. Experimental findings have approved that saffron may be improve hyperglycaemia by multiple mechanisms such as increase in glucose uptake by peripheral tissues [34], decrease in intestinal absorption of glucose [35], inhibition of renal reabsorption of glucose [36], inhibition of insulinase activity [37] and inhibition of gluconeogenesis [38]. Also, it has been defined that saffron improved insulin resistances and increased insulin sensitivity in diabetes [39]. In another study, administration of the ethanolic saffron extract (20, 40 and 80 mg/kg) could reduce the levels of fasting blood glucose (FBG) by increasing the number of pancreatic beta cells and induction of insulin secretion in alloxan induced diabetic rats [25].

Diabetic nephropathy (DN), a kidney disease with high prevalence that resulted from diabetic microvascular injuries, recognized as a common cause of end stage renal disease [40]. It has been suggested that oxidative stress directly contributes to the development and progression of DN [9]. Free radicals facilitate tissue damages through lipid peroxidation, alteration in the activity of antioxidant enzymes, $NF - \kappa \beta$ activation and induction of apoptosis [32]. It was reported that chronic hyperglycaemia in DM induces over production of ROS along impairs in the activity of antioxidant enzymes such as SOD, GPx and CAT [41]. Our data showed a significant reduction in the activity of SOD and consequently increase in MDA levels in kidney tissues of diabetic rats compared to healthy groups (Group 1 and Group 2) (Figure 2). Reduced enzyme activities may be due to the non-enzymatic glycation of enzymes or structural damages by over production of ROS in hyperglycaemia condition. So, antioxidant therapy has considered as a beneficial choice to inhibition of hyperglycaemia induced oxidative stress. Animal studies have approved the ability of natural antioxidants such as vitamin E, combined vitamin C - E and β -carotene to decrease of oxidative stress in diabetes [42]. The present study has revealed that weekly saffron administration that has antioxidant activity can improve enzymatic antioxidant systems and reduces lipid peroxidation in kidney tissues of diabetic rats. SAE significantly increased the activity of GPx, SOD and CAT in the kidney tissues of diabetic rats in comparison to other groups (Figure 2). It has been suggested that major saffron carotenoids such as crocin and crocetin are responsible for its antioxidant activity. Previous data reported that saffron as an antioxidant could stabilize cell membranes, decrease lipid peroxidation, and scavenge ROS. Also, saffron as a modulator can affect gene expression and induces the expression of antioxidant enzymes genes [43-46]. Our observations are compatible with Samarghandian et al. who reported that the ameliorative effect of saffron on decreased activity of SOD, CAT and GPx in brain tissues of diabetic rats [24]. Antioxidant enzymes protect the cell from free radical's damages. The antioxidant activities of SOD, GPx and CAT are related to whose ability to remove of superoxide, hydrogen peroxide and hydroxyl radicals. In the present study SAE improved oxidative stress in the kidney tissues of diabetic rats due to increase in antioxidant enzymes activities.

Evaluation of important enzymes activities in kidney tissues showed the significant increase in the activity of aminotransferases (ALT, AST), LDH and ALP in untreated diabetic rats compared to healthy Group 1 (Table 2). While, the activity of GGT reduced significantly in group 3 in comparison to Group 1. These alterations in the activity of mentioned enzymes in untreated diabetic rats may be due to the metabolic abnormalities or cellular injuries [47]. Zafar, et al. have reported that the increase in AST, ALT, ALP and PChE activities in the kidney tissues of STZ

treated rats due to the subtle membrane changes that allow to passage of intracellular enzymes to the extracellular space [48]. Also, Rogers et al. have shown decrease in the mitochondrial activity of AST and versus 3-4-fold increase in its cytoplasmic activity in diabetic rats [49]. ALP is a membrane bound enzyme and it is often used as a marker for assessment the integrity of plasma and endoplasmic membranes [50]. In the present study, its activity was increased in the kidney tissues of diabetic rats. Leibovitch, et al. reported that the increase in ALP activity in DM may be due to the damage of brush border membrane of renal tubular cells [51]. Our data has shown SAE modulate the activity of above enzymes that changed by STZ treatment. About of ALT, AST, ALP, and LDH, their activities nearly normalized with SAE treatment. These results are in accordance with Rahbani, et al. study that revealed the hepatoprotective effect of ethanolic extract of saffron in STZ-induced diabetic rats due to decrease in the activity such as GPx, SOD and CAT [52]. In addition, daily administration of ethanolic extract of saffron can stabilize the hepatocellular membrane and inhibit intracellular enzyme permeability [52]. This finding is in line with our results.

Our histopathological study showed infiltration of lymphocytes in the interstitial spaces, glomerular hypertrophy and vacuolated cytoplasm of tubular epithelial cells in untreated diabetic group. The injuries in SAE treated diabetic group was less (Figure 3). These results are in line with the finding of Zafar, et al. that reported the alteration of kidney morphology in STZ diabetic rats [48]. In addition, the results of Komolafe, et al. revealed that *Psidium guajava* extract with antioxidant activity has improved hypertrophy in the glomeruli and morphological alteration of kidney tissues in STZ induced diabetic rats [53]. Therefore, histopathological results in the present study confirmed the biochemical results and the protective effect of SAE on kidney tissues of diabetic rats.

CONCLUSION

In conclusion, the renal protective effect of saffron aqueous extract could be associated primarily with its antioxidant activity due to its major phytochemicals including crocins. Radical scavenging activity and hypoglycaemic property of saffron make it as a suitable candidate to prevent from the development or progression of diabetic renal disease. Saffron ability to normalize the activity of kidney functional enzymes approved its protective effect on diabetic renal disease in this study.

DECLARATIONS

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Conflict of interests

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

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