The local Propolis as protective and therapeutic modulator on some biochemical parameters in diabetic nephropathic rats.

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الخلاصة:
الهدف من هذه الدراسة هو التحقق من تأثير المستخلص الايثانولي للعكبر المحلي من خلال التجريع اليومي على مستوى الكلوكوز، النظام الدفاعي المضاد للأكسدة في الجسم و كذلك تأثيره على وظيفة الكلية في الجرذان المختبرية المصابة بالفشل الكلوي نتيجة داء السكري المحفز بواسطة الستربتوزوتوسين (60ملغم/ كغم).
اجريت الدراسة على خمسة وسبعون جرذا (150- 160) غم قسمت الى خمس مجاميع : المجموعه الطبيعيه ، المجموعة المحفزة بداء السكري، مجموعه طبيعيه تجرع بالمستخلص الايثانولي للعكبر المحلي يوميا مدة 6 اسابيع، مجموعه تجرع بالمستخلص الايثانولي للعكبر المحلي لمدة 3 أسابيع ثم يتم تحفيز داء السكري لمدة 3 اسابيع اخرى و مجموعه طبيعيه يتم تحفيز السكري بعد 3 اسابيع من بداية التجربة ثم تجرع بالعكبر المحلي لمدة 3 اسابيع بعد التحفيز.
بعد 6 اسابيع جمعت عينات مصل الدم من الجرذان تحت التخدير لتعيين التغيرات البايوكيميائيه في المجاميع المختلفه. قيمت الحاله التأكسديه للمجاميع من خلال قياس فعالية الكلوتاثيون بيروكسيديز وتركيز الكلوتاثيون. وكذلك تم تعيين مدى الأصابة بالفشل الكلوي عن طريق قياس اليوريا والكرياتينين في مصل الدم. لوحظ في نهاية الدراسة انخفاضا معنويا ($p<0.05$) في وزن الجسم و فعالية الكلوتاثيون بيروكسيديز و تركيز الكلوتاثيون، و زيادة معنوية ($p<0.05$) في مستوى الكلوكوز في الدم وزيادة تركيز اليوريا و الكرياتنين في مصل الدم، بالنسبة للمجموعة المصابة بالسكري.
بينت النتائج أن المستخلص الايثانولي للعكبر المحلي كان له تأثيرا معنويا ($p<0.05$)في تقليل مستوى الكلوكوز و اليوريا و الكرياتينين ، و حدوث زيادة معنوية($p<0.05$) في فعالية الكلوتاثيون بيروكسيديز و تركيز الكلوتاثيون وبالتالي حماية الكلية و تعديل فعالياتها من خلال تقليل تأثيرداء السكري و مضاعفاته كالفشل الكلوي.

Abstract:

The aim of the present study was to investigate the effect of daily oral administration of local Ethanolic Extract of Propolis (EPP) on blood glucose, antioxidant defense system and kidney function in streptozotocin (STZ)-induced diabetic rats to show the ameliorating and partly curative effects in STZ-induced rats(60mg/kg) intrapretonial (i.p). Seventy five rats (150-160gm) were divided into 5 groups, normal rats, diabetic rats, normal treated with local EEP for 6 weeks, pretreated with local EEP for 3 weeks then induced diabetic in rats for 3 weeks and the last group, after 3 weeks(normal) induced diabetic in rats then post- treated with local EEP for 3 weeks. The dose of EEP 200mg/kg for the last three groups, and groups 2, 4 and 5 were induced diabetes in the same time at the end of the first 3 weeks.

After 6 weeks , serum of samples were collected from anesthetic rats to determined the biochemical alterations.

Antioxidant status in rats were estimated by determined the Glutathione(GSH) concentration, and Glutathion peroxidase (GPx) activity, and also measured renal function by the tests of Serum Urea and Creatinine concentrations, the result of this research indicated the effect of local EEP as protective or therapeutic in this experiment, the diabetic group showed decreased significantly($p<0.05$) in body weight gain, increased($p<0.05$) blood glucose, decreased significantly($p<0.05$) activity of GPx and GSH concentration and increased significantly($p<0.05$) serum Urea and Creatinine concentrations, as well as local EEP was moderated and effected significantly decrease($p<0.05$) glucose level, Urea and Creatinine concentrations,

increased significantly($p<0.05$) activity of GPx and GSH concentration, as a result, it led to protect the kidney from damage by moderated their functions.

Introduction:

Diabetes mellitus is a metabollic disorder characterized by hyperglycemia because of defects in insulin , insulin action , or both , the chronic hyperglycemia of diabetes is associated with dysfunction and failure of many organs , specially the eyes , nerve and kidney [1].Diabetic nephropathy (DN) is the most complication of the two kinds of diabetes mellitus[2]. It is the major reason of end-stage renal disease[3].

There are several mechanisms that cause development (DN), such as increased formation of advanced glycation end products (AGEs), the activation of protein kinase C (PKC) isoforms [4], the activation of polyol pathway mechanism[5], and glucose auto oxidation, these mechanisms induced diabetic complication [4].

These not only generate ROS but also attenuate antioxidant mechanisms creating a state of oxidative stress [6]. The pathophysiology of diabetic nephropathy can be viewed as a sequence of events evolving in a stepwise pattern, where it starts with endothelial cell dysfunction (ECD) and ends with end-stage renal failure. However, ECD is preceded by glomerular hyperperfusion and hyperfiltration [7].

Propolis also is an adhesive, dark yellow to brown colored balsam that smells like resin. The composition of propolis depend on its botanical and thus also on its geographical origin [8]. Propolis contains about 300 constituent [9]. Recently, propolis has gained popularity in connection with oxidative stress [10], and prevent disease such as inflammation, heart disease, diabetes and even cancer [11]. Because of the broad spectrum of biological properties, there is a great interest in its biological activities, and showed that flavonoids concentrated in propolis are powerful antioxidants which are capable to scavenge free radicals [12], thought to be responsible for many of its biological and pharm logical activities [13].

Materials and methods:

Experimental animals:

Seventy-five Spargue- Dawley male rats weighting between 150 ± 10 gm were used. Rates were housing in the animal house of Veterinary medicine college of Al - Qadisiyah University.

The animals were allowed to acclimatize for one week, before the experiment. The animals were housed in polypropylene cages inside a well-ventilated room. Each cage consists of not more than five rats, they were fed a standard commercial pellet diet and water. They were maintained under standard laboratory condition of temperature 22-25 c^o.

Preparation of local Ethanolic Extract of Propolis (EEP)

The samples of crude local propolis were cut into small pieces by using medical mortar, then grinded by using of electrical grinder to a powdered suitable practical size following grinding, the powdered crude propolis was sifted to ensure the proper practical size, where in sieving the material is passed through a sieve of suitable mesh size giving two fractions. The fraction passing the sieve consist of particles with a size smaller than or corresponding to the mesh size, and the remaining fraction consists of coarser particles which are returned to the electrical grinder for further grinding.

By methods presented by (Al-Mohana, 2004 and Yaghobi et al,2007)[14,15] prepared the pure local EEP, the yield of extraction of propolis was determined from the proportion of dry weight of extracted propolis to that of crude propolis using the following formula.

$$\text{The yield} = \frac{\text{Weight of extracted propolis}}{\text{Weight of crude propolis}} \times 100$$

Before using, (2) grams of propolis extract was dissolved in (4) ml of absolute ethanol by using of vortex mixer until complete dissolving occurred, then the volume was completed to 100 ml by adding of distilled

water to obtain 2% (W/V) milking solution. The final concentration of ethanol in this milky solution didn't exceed 5% which had no effect on *in vivo* and *in vitro* experiment according to what stated by[16]. Primary Chemical test of EEP Test for Alkaloids, Flavonoids, Tannins, Resins, phenols , Terpenoids , Coumirians and Saponin was done to determine the constituents of local propolis[17].

Preparation STZ solution

STZ was by the method of (Sachin et al 2009) [18].

Induction of diabetes:

Diabetes mellitus was induced in the overnight fasted rats by a single (i.p) injection of streptozotocin , at a dose of 60 mg/ kg body weight**.** STZ was dissolved in citrate buffer (pH 4.5) and freshly prepared before injection. Hyperglycemia in rats followed up for 72 hours, by using blood test strips depend on the method by(Stedman's. 2006)[19] that used with Accu-Chek Active meter, hyperglycemic rats were confirmed by the elevated glucose levels in plasma on day 5 after injection. Male rats with blood glucose concentration more than 250 mg/ dl were considered as diabetic[20], and used for evaluation the anti-hyperglycemic effect of the ethanolic extracts of propolis.

Experimental Design

All rats were randomly divided into five groups, fifteen rats for each group. Animals of all groups were administered as follows:

(G1)Control (intact): Drenched orally with distilled water at a dose of (10ml/ kg. b. w) once time a day for six weeks, including stopped after the end of week 3 for 5 days, when injected with a single dose of citrate buffer (3ml/kilo. b. w).

(G2) Diabetic nephropathy control: Rats were received distilled water (10ml/kg. b. w) for three weeks, then after overnight fasting (deprived of food for 16 hours) diabetes was induced by i.p injection of a single dose of STZ (60mg/ kg) by using insulin injector. After five days to ensure of induction diabetes, rats drenched distilled water at a dose level (10ml/ kg. b. w) daily for three weeks again.

(G3) Treatment : Drenched local EEP at dose of (200mg/ kg. b. w) [21, 22], in average of (10ml/kg. b. w) from freshly prepared milky solution for three weeks, then injected with a single dose of citrate buffer (3ml/kilo. b. w), after stopped for 5 days, was continued with gavage of local EEP for three weeks again.

(G4) Pre-treated : Rats were pretreated with local EEP at a dose of (200mg/kg. b. w) for three weeks, after that they were injected with a single dose of STZ (60mg/ kg) once time to induce diabetes , then after 5 days was continued drenched orally with distilled water for three weeks.

(G5)Post- treated : Rats(post-treated) were received distilled water at (10ml/ kg. b. w) for three weeks, after that received local EEP at dose level (200mg/ kg. B. W) daily for three weeks, starting after 5 days of STZ (60mg/ kg. b. w) injection and diabetes induced.

Every three days body weights have been registered by electric balance, and blood glucose level of rats (group 2,4 and 5) were taken after induction diabetes every three days, by using the blood glucose monitoring instrument, type Accu-chek Active meter(blood was withdrawn from tail-vein).

In 49 day of the beginning of experiment, all groups were withheld from food until the following day for 12 hours before blood sample collection. All rats animals were lightly anesthetized with ketamine (50mg/ kg) injected i.P[23]. After sedation, each rats was fixed on the rat dissecting table, and mid line thoracic incision was made to the lower abdominal posterior aorta, which the greater vessels were easily exposed. Blood was directly collected and animals were sacrificed.

Serum preparation

Blood was collected in test tubes with cap and allowed to clot (20) minutes, then serum

was separated by centrifugation at (4000 rpm, 0.894xg) at 37C° for (10) minutes [24]. The separated serum of each animal was subdivided nearly into (6) samples using of appendoff tubes (500µl) and kept at deep freezer until using for assessment of the biochemical parameters.

Assessment of Glutathione Peroxidase.

by Flohe and Gunzler method, 1984[25]. The color that develops is read against reagent blank at the range 420 nm on a spectrophotometer Aple PD-303 UV.

Assessment of Serum Glutathione.

By method of Burti and Ashwood,1999. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration[26].

Determination of serum urea [27].

By using serum urea kit depend on the method of Chany, A.L. 1992. The absorbance was done at 600 nm.

Determination of serum Creatinine [28].

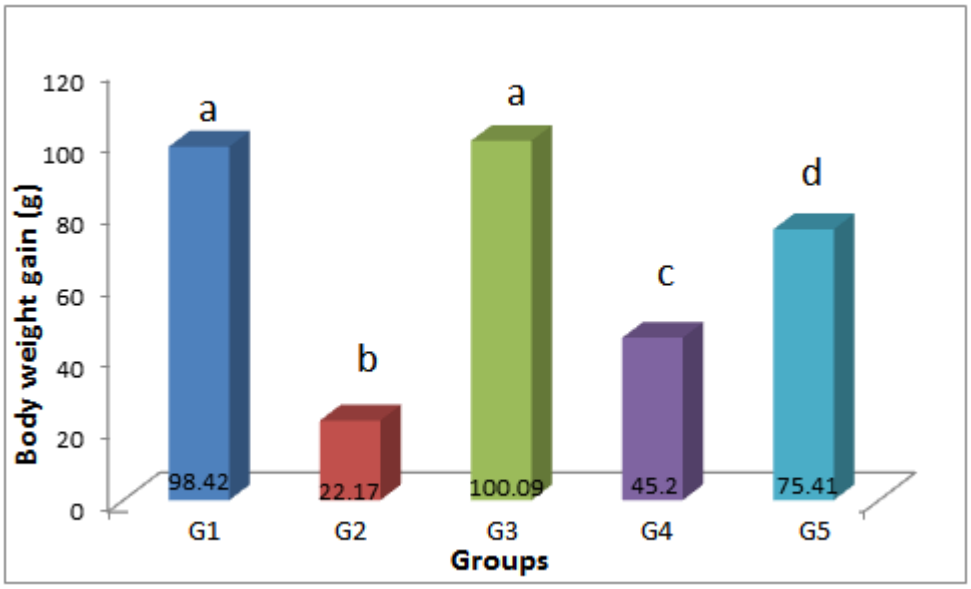
By using serum Creatinine kit depend on the method of Klin. Z., 1974and Jaffe. J., 1886. The absorbance was measured at 500 nm.

The yield of local EEP is obtained in this study, after complete dryness was viscous and dark brown material. The yield of local EEP according to different part of Al-Diwaniya province is range between 38%- 42%.

The primary chemical test showed positive result for flavonoids, tannins, resins, terpenoids, phenols, alkaloids, saponin, but negative result for Coumarines.

About body weight of rats, all groups of rats was showed nearly the same gradual increased in body weight until the day 21,but after induction the diabetes, the difference in the body weight gain was appearance. G1(control) and G3 (treatment1) animals showed a gradual increased in body weight, while G2 (diabetic group) was associated with marked and gradual reduction in the body weight gain reaching 21% compared to that of the G1(control), In G4(pretreated EEP) and G5(post- treated EEP) the decrease in body weight gain was suppressed, and the reduction in the body weight recording 14% and 7%), respectively compared to G1(control).The gain in the body that show in figure(1)

RESULTS



Figure(1): Effect of local EEP on body weight gain(g) after induced diabetic rats. G1= Intact control. G2= Diabetic rats. G3= Treatment with local EEP, non-diabetic group. G4= Diabetic with Pre-

treated with EEP. G5= Diabetic with post- treated with local EEP. *Different letters represent significant difference between groups(p<0.05).*Similar letters represent insignificant difference between groups.

Biochemical analysis

Blood glucose level In STZ-diabetic rats, there was a significant ($p < 0.05$) increase in blood glucose levels. The administration of local EEP to the diabetic rats whether pretreated or post- treated significantly($P<0.05$) reduced blood glucose level when compared with diabetic group, this reduction was not enough to reach normal value, but it was still significantly($P<0.05$) higher when compared with the control group. On other hand , from table (1), it was clear that the level of blood glucose was decreased significantly($p<0.05$) in rats post-treated with local EEP more than pre-treated , but it was significantly($p<0.05$) higher than control group.

Table(1): Effect of local EEP on blood glucose level in STZ-induced diabetic rat. .G1= Intact control. G2= Diabetic rats. G3= Treatment with EEP, non-diabetic group. G4= Diabetic with Pre- treated with EEP. G5= Diabetic with post- treated with EEP. *Different letters represent significant difference in the same group(p<0.05).*Similar letters represent insignificant difference in the same group.

Days	G1(control) Mean∓SE	G2(Diabetic Rats) Mean∓SE	G3 (EEP alone) Mean∓SE	G4(pre-treated with EEP) Mean∓SE	G5 (post-treted with EEP) Mean∓SE
21	93.73∓1.09 **a**	93.06∓1.66 **a**	92.66∓1.78 **a**	91∓1.78 **a**	91.26∓1.79 **a**
27	94.60∓1.29 **a**	485.13∓17.65 **b**	95.73∓1.25 **a**	289.12∓11.35 **c**	446.66∓16.17 **b**
30	96.43∓2.05 **a**	506.5∓15.58 **b**	94.2∓1.56 **a**	296 ∓ 10.01 **c**	395.61∓15.79 **d**
33	91.8 ∓ 1.55 **a**	508.14∓17.38 **b**	91.4 ∓ 1.74 **a**	301.86∓12.26 **d**	366.46∓13.89 **d**
36	92.3 ∓ 1.59 **a**	505.85∓17.12 **b**	87.94∓2.99 **a**	294.73∓10.74 **c**	354.26∓14.25 **d**
40	92.46∓1.60 **a**	512.56∓13.56 **b**	90.8 ∓ 2.01 **a**	302.2∓10.65 **d**	324.13∓14.28 **d**
43	96.1 ∓ 2.11 **a**	508.68∓14.98 **b**	91.81.681 **a**	301.21∓10.65 **d**	299.86∓12.4 **c**
46	91.7 ∓ 1.27 **a**	514.16∓16.31 **b**	92.5 ∓ 1.69 **a**	302.71∓9.79 **d**	275.53∓9.44 **c**
50	90.8 ∓ 1.79 **a**	518.81∓16.33 **b**	92.7 ∓ 2.04 **a**	301.13 ∓ 11.4 **d**	250.66∓6.24 **c**

In this study, were noticed significant decrease ($P < 0.05$) in antioxidant enzymes and non- enzymatic factor, found in the diabetic group as compared to the control group, GPx activity (43%), GSH concentration (51%), but in G4(pretreated EEP) and G5(post- treated EEP), there is significant($P < 0.05$) improved in GPx activity (79%), (88%), GSH concentration (66%), (84%) respectively compared to G1(control), while there is significant increased ($P<0.05$) in GPx activity and GSH

concentration compared to G2 (diabetic group). In G3(treatment) there is significant increased($P<0.05$) in the GSH concentration, but there is no different in GPx activity compared to G1, that show in table (2).

Also in this study showed some parameter that test the function of kidney, increased significantly($P<0.05$) in the level of serum Creatinine, serum Urea concentrations in diabetic rats (G2) compared to control (G1). In the treatment group(G3), there is no significant difference in Urea and Creatinin concentrations compared to G1. In Pre-treated (G4) and Post-treated (G5) groups, there is significant decrease ($P<0.05$) in serum Creatinine and Urea concentrations in G4and G5 compared to G2 (diabetic group), but the results of G4, G5 were still significantly higher than control rats (G1) in the level of creatinine and urea . The results of post- treated is the best compared with pre-treated, that show in table (3).

Table (2). Effect of local EEP on serum antioxidants in STZ-induced diabetic rats. .G1= Intact control. G2= Diabetic rats. G3= Treatment with EEP, non-diabetic group. G4= Diabetic rats with Pre- treated with EEP. G5= Diabetic rats with post- treated with local EEP. *Different letters represent significant difference between groups($p<0.05$).*Similar letters represent insignificant difference between groups.

Parameters Mean∓SE	G1(control) Mean∓SE	G2(Diabetic Rats) Mean∓SE	G3 (EEP alone) Mean∓SE	G4(pre-treated with EEP) Mean∓SE	G5(post-treted with EEP) Mean∓SE
GPx Activity U/L	7.08∓0.04 **a**	3.01∓0.09 **b**	7.28∓0.02 **a**	5.66∓0.07 **c**	6.28∓0.06 **d**
GSH Conc. µmole/L	2.24∓0.02 **a**	1.16∓0.03 **b**	2.62∓0.02 **c**	1.47∓0.02 **d**	1.88∓0.01 **e**

Table (3). Effect of local EEP on serum biochemical parameters reflect renal function in STZ-induced diabetic rats. .G1= Intact control. G2= Diabetic rats. G3= Treatment with EEP, non-diabetic group.G4= Diabetic with Pre- treated with EEP. G5= Diabetic with post-treated. *Different letters represent significant difference between groups($p<0.05$).*Similar letters represent insignificant difference between groups.

Parameters	G1(control) Mean∓SE	G2(Diabetic Rats) Mean∓SE	G3 (EEP alone) Mean∓SE	G4(pre-treated with EEP) Mean∓SE	G5 (post-treted with EEP) Mean∓SE
Creatinin Conc.mg/L	0.55∓0.01 **a**	2.62∓0.06 **b**	0.48∓0.01 **a**	1.39∓0.03 **c**	0.94∓0.03 **d**
Urea Conc. mg/dL	20.37∓0.16 **a**	50.23∓0.57 **b**	18.99∓0.20 **a**	40.35∓0.71 **c**	34.98∓0.64 **d**

Discussion:

The yield of local EEP according to different part of Al-Diwaniya province is range between 38%- 42%. This yield agreement with documented by previous study as (Cunha et al. 2004)[29] they obtained

38.23%- 40.43% yield of EEP after extraction of Brazilian propolis with absolute alcohol by maceration, and also little more than the yield of stated by (Al-Mohana:2004)[14] who obtained 33% yield of EEP from propolis that obtained from different Iraqi provinces.

The presence of flavonoids, tannins, resins, phenols, terpenoids and saponin in propolis came in agreement with that reported by the researchers as (Koo and Park;1997)and (Munzo et al;2001)[30,31], and the absent of Coumarines in EEP in this study is agreement with stated by(Al-mohana.2004)[14], but came in contrast with the result of (Marcucci et al. 2001)[32]

The presence of alkaloids came in agreement with that reported by the researchers as (Robert et al.2012 and Foket et al. 2010)[33, 34], but that disagreement with the result of (Al- Mohana.2004)[14] who noted the negative results for the presence of alkaloids. This contrast might be attributed to the variation in plant sources in areas of propolis as well as the difference in season of propolis collection. All these constituents in propolis known to show medicinal activity as well as exhibiting physiological activity.

The results about body weight gain agree with the research of (Walaa et al 2012)[35] ,but disagree with the report by (Denli et al. 2005)[36] that showed increase in body weight when treatment propolis to Coturnix bird. but in diabetic group propolis treatment showed significant amelioration in body weight, propolis has a strong antioxidant and free radical scavenging effect[37], this finding suggests that propolis may improve the disturbed metabolism associated with diabetes.

Our results about the concentration of blood glucose agree with (Matsui, et al 2004)[38] who demonstrated that administration of propolis extract in rats had a potent antihyperglycemic effect. the presence of flavonoids and polyphenolic components as main active ingredients having potent antioxidant activities [39, 40], that Enhancement of antioxidant defense systems in pancreatic tissue[41]. Propolis also, contains tannin that can stimulated secretion insulin, that showed in reported of the researchers (Gray et al.2000)[42], the study indicated that tannic acid in the aqueous extract of the elderberry plant worked to stimulate insulin secretion.

The reduction of the enzymes GPx, is due to decrease in GSH concentration which leads to an increase in oxidative stress. However, it is important to note that elevation of blood glucose level generates oxidative stress, which contribute to increase in glutathione utilization [43].

El- Sayed et al[41] mentioned that there is marked significant reduction in the antioxidant parameters as GSH in STZ induced diabetes rats, these results from different researchs were conformed with this study about effect of diabetes on antioxidant system, and may suggested a strong antioxidant effect of propolis which can occurrence of diabetic nephropathy in diabetes mellitus[44].The increase activity of antioxidant enzymes because the propolis reactivated the antioxidant enzymes and restored GSH level and GPx activity, that linked with GSH level, which in turn increased the detoxification of active metabolism of STZ and ROS. Flavonoids and their esters are the pharmacologically active molecules of propolis and have been hypothesized to influence the antioxidants activity of propolis. At least 38 flavonoids have been found in propolis [45& 46].

Hyperglycemia is a factor in the development and progression of the complication of diabetes mellitus [47], and induces the elevation of the blood urea and serum creatinine levels in diabetic rats, which are considered as significant markers of renal dysfunction and indication of the development of diabetic nephropathy in rats [48,49]. Blood Urea and Creatinine concentrations declined significantly in the propolis- treated groups. These results may indicate that propolis can attenuate renal

damage in diabetic rats, in agreement with these finding (Yamabe et al.2006) reported that antioxidant and good control of diabetes led to improved renal function[50]. So the report by(Oktem et al. 2005) [51] maintained that, Caffeic acid phenethyl ester (CAPE), a biological active compenent of propolis was found to improve renal function.

Conclusion

The result of the present study demonstrates the effect of propolis as antioxidant which can decrease metabolic disturbances and oxidative stress that are associated with diabetes. Propolis may delay the onset and progression diabetic nephropathy and delay the occurrence of diabetes- associated renal function impairment. From the data obtained, it is concluded thet post-treatment and pre-treatment with local EEP produced a significant anti-hyperglycemic effect. Furthermore, EEP is capable of improving hyperglycemia and the impaired kidney function in STZ-induced-diabetic rats.

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