Evaluation of the Antioxidant Activity of the Polyherbal (*Conocarpus lancifolius* L., *Capparis spinosa* L. and *Dodonaea viscosa*) Extracts and Assessment of the Hypoglycaemia Effect in Diabetic Mice

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Abstract

Diabetes mellitus, often referred to simply as diabetes, is a chronic metabolic disorder due to the relative deficiency of insulin secretion and varying degrees of insulin resistance. It is characterised by high circulating glucose. Excessive levels of either molecular oxygen or Reactive Oxygen Species (ROS) lead to an imbalance in the body's normal oxidative metabolism that to leads to high glucose levels in the blood (hyperglycaemia), resulting in metabolic disturbances (oxidative stress) and chronic complications in diabetes. The present study aims to estimate the antioxidant activity in diabetic mice induced via alloxan of a combination of three types of plant leaves (*Conocarpus lancifolius* L., *Capparis spinosa* L. and *Dodonaea viscosa*). The total phenolic content using Folin-Ciocalteu reagent and the antioxidant activity utilizing 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay were estimated. The effect of polyherbal formulation leaves extracts on serum glucose level was done on forty-two albino mice divided into six groups and treated with polyherbal extracts and metformin. The results of total phenolic content in the polyherbal leaves extracts was observed (15.52 mg\g) in the aqueous extracted samples, while the total phenolics content was (46.97mg\g) in the methanolic extract, and the antioxidant activity showed the methanolic extract was the highest free radical scavenging activity (93.28%) than the aqueous extract (86.77%) in 10 mg/ml and approach to the artificial antioxidant Butylated hydroxytoluene (BHT) which was (93.67%). This study showed both extracts of polyherbal leaves did not induce lethality in mice when administered orally at a dose of 2000 mg/kg. on the other hand, Diabetic mice treated with methanolic extract at doses 200 and 400 mg/kg showed a significant decrease ($p<0.01$) in serum glucose level after 35 days, which was 113.66 and 107.66 mg/dl respectively, and the aqueous extract was 124.66 and 117.00 mg/dl respectively when compared with the control positive group (324.00 mg/dl).

Keywords: *polyherbal, total phenol, antioxidant, hypoglycaemia, diabetic mice.*

Introduction

Diabetes mellitus is a non-infectious endocrine disorder which is characterized by the disturbance in the metabolism of carbohydrates and associated with hyperglycaemia[1]. It is linked with the developing of various serious diseases like micro-vascular (nephropathy, retinopathy, nephropathy) and macro-vascular (peripheral vascular disease and coronary heart diseases)[2, 3]. Diabetes mellitus is known as diabetes which was observed as diseases related to "sweet urine" and muscle loss. Glucose blood levels are maintained by insulin which is a hormone released from the pancreas. When these level increases, insulin is produced from the pancreas and maintained the level of glucose. In diabetic patients, the production of insulin is absent or less which causes hyperglycaemia [4].

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Treatment of Diabetes mellitus without any adverse effects is still the biggest question for medical practitioners. Medicinal plants containing secondary metabolites such as phenolic, anthocyanin, and flavonoids compounds have been used as alternative therapeutic tools to treat many diseases throughout medical history. Several types of plant extracts or plant-derived molecules have been investigated for their potential as antioxidant sources against several diseases [5, 6, 7]. In addition, plant-based natural antioxidants are preferred to synthetic ones due to their good safety profiles [8]. Therefore, there is growing interest in finding natural compounds that could prevent oxidative damage underlying the pathogenesis of many diseases [9]. The aim of the study is to assessment of the efficiency of polyherbal leaves extracts as antioxidant in diabetic mice induced via alloxan.

Materials and Methods

Chemical reagents

The chemical reagents DPPH (2,2-diphenyl-1-picrylhydrazyl), Butylated hydroxytoluene (BHT), ascorbic acid, gallic acid monohydrate (3,4,5-trihydroxybenzoic acid), sodium carbonate and metformin were purchased from Sigma aldrich chemicals (Sigma-Aldrich, Germany). Folin Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), alloxan (BDH, England).

Collection of plants

Three species of plant leaves were collected from the trees in the gardens of the University of Baghdad. The plant leaves were identified as (*Conocarpus lancifolius* L., *Capparis spinosa* L. and *Dodonaea viscosa*) by the specialist, Department of Biology, College of Science, University of Baghdad. The plant leaves washed with water to remove the dust and soil deposits and dried at room temperature until complete removal of moisture content, each dried plants were crushed using a grinder and stored at -20°C for further analysis.

Polyherbal preparation

The polyherbal leaves extract was prepared by mixing the dried extracts of the plant leaves (*Conocarpus lancifolius* L., *Capparis spinosa* L. and *Dodonaea viscosa*) in the ratio of 1: 1: 1 respectively.

Preparation of aqueous extract

Water extract was prepared according to N'Guessan *et al*. [10], macerated 100 grams of plant leaves residue in 700 ml of distilled water for 72 hours with continuous shaking. Then the mixture was vacuum filtered through Whitman No. 1 paper. The filtrate evaporated to dryness under vacuum at 50°C by a rotary evaporator to eliminate water. The resulting extract stored in amber glass vials at 4 °C until analyzed.

Preparation of methanolic extract

The methanolic extract was prepared according to AACC [11] by using a Soxhlet apparatus. 100 grams of plant leaves residue was put in a thimble and 700 ml of 70% methanol was added within 40-60 °C for 6 hours. The solution was filtered through a filter paper Whitman No.1 and evaporated to dryness under vacuum at 40°C by a rotary evaporator to get rid of methanol; the extract was stored in amber glass vials at 4 °C until analyzed.

Determination of total phenolic contents

Total phenolic content of *Moringa oleifera* extracts were determined spectrophotometrically using the Folin-Ciocalteu method described by [12]. 2 ml of Folin-Ciocalteu reagent (diluted 10 times) was mixed with 1.6 ml of 7.5% sodium carbonate solution and 0.4 ml of *Moringa oleifera* extracts. The volume was completed to 5 ml by adding distilled water. The tubes were covered with parafilm for 30 min. at room temperature, and then the absorbance was read at 760 nm spectrophotometrically.

Evaluation of the Antioxidant activity DPPH assay

According to Ogunmoyole *et al*. [13], the antioxidant activity of the prepared polyherbal methanolic and aqueous leaves extracts was conducted. 5 ml of a freshly prepared 0.004 % of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was mixed with 50 µl of different concentrations (0.625, 1.25, 2.5, 5 and 10) mg/ml, which were prepared by dissolving 0.1 gram of the polyherbal extract in distilled water then the volume was completed into 10 ml to make the working solution 10 mg/ml, and

serial two-fold dilutions of the polyherbal extract were prepared to make the concentrations 10-0.625 mg/ml. The absorbance of each dilution, after 30 minutes, was measured at 517 nm. Butylated hydroxytoluene (BHT) and vitamin C were used as a positive control. All tests were performed in triplicate. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as:

% Reduction = (Abs DPPH – Abs Dil.) / Abs DPPH × 100

Where:

Abs DPPH = average absorption of the DPPH solution

Abs Dil. = average absorption of the three absorption values of each dilution.

With the obtained values, a graphic was made using Microsoft Excel. The EC_{50} of each extract (concentration of extract or compound at which reduced 50% of DPPH) was taken from the graphic.

Experimental animals

Forty-Two male albino mice weighing 27-32 grams were obtained from Biotechnology Research Center, Al- Nahrain University. They were kept in standard conditions, the temperature about 22 °C, 12 hours light/dark cycle. They were left for two weeks for acclimatization with the experimental conditions. Standard pellet diet and water were provided daily.

Determination of acute toxicity of polyherbal extracts

Acute toxicity of the polyherbal preparation was carried according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423. A group of 30 adult healthy albino mice of either sex weighing 27-32 grams was divided into five groups (six mice / group) for each extract (aqueous and methanolic). All groups were treated orally with doses of 100, 250, 500, 1000 and 2000 mg/kg of polyherbal preparation to study the acute toxicity. The animals were then observed for 3 hours for general behavioural, neurological, and autonomic profiles and every 30 min for the next 3 hours and finally for mortality after 24 hours [14].

Selection of doses

To estimation the antidiabetic activity, two-dose levels were chosen (200 and 400 mg/kg body weight) in such a way that the first dose was approximately one-tenth of the maximum dose during acute toxicity studies and the second high dose was twice of the first dose.

Induction of experimental diabetes

Blood glucose levels (baseline) were tested before the treatments. Diabetes was induced in all mice (except normal control group) by a single dose of alloxan monohydrate (150 mg/kg body weight) intraperitoneally to overnight fasted mice. After 1 hour of alloxan administration, the animals were fed with standard pellets and water. Seventy-two hours later of alloxan administered, blood glucose was measured by glucometer which was collected from the tail vein from the mice. Mice showing fasting blood glucose levels (>250 mg/dl) were selected for the study [15].

Evaluation of the antidiabetic activity of the polyherbal extracts

The Diabetic mice were randomly divided into seven groups with six animals in each group. The single dose of each extract and drug was administered once daily by oral for 35 days continuously as follows:

Group 1: This group served as a negative control in which the mice received normal feed and distilled water.

Group 2: This group was a positive control for alloxan (150 mg/kg BW).

Group 3: Diabetic mice of this group treated with standard drug metformin (150 mg/kg BW/day).

Group 4: Diabetic mice of this group treated with the polyherbal methanolic extract (200 mg/kg BW/day).

Group 5: Diabetic mice of this group treated with the polyherbal methanolic extract (400 mg/kg BW/day).

Group 6: Diabetic mice of this group treated with the polyherbal aqueous extract (200 mg/kg BW/day).

Group 7: Diabetic mice of this group treated with the polyherbal aqueous extract (400 mg/kg BW/day).

Collection of Blood

Every week blood glucose levels were measuring. The blood samples were collected from the tail vein of each mice of the group as a drop. The drop was then immediately placed on the strip of the glucometer to find the glucose level quickly.

Statistical Analysis

The Statistical Analysis System-SAS program [16] was used to detect the effect of difference factors in study parameters. Least significant difference-LSD test was used to significant compare between means in this study.

Results and Discussion

Total phenolic content of polyherbal leaves extracts

The polyherbal leaves *extracts* were evaluated by using Folin-Ciocalteu reagent for the determination of total phenolic contents. The results showed that the methanolic extract had the highest total phenolic content than the aqueous extract as shown in Table (1). Polyphenols are known for their strong antioxidant properties, their activity is based on scavenging free radicals and reactive oxygen/nitrogen species, the reduction of oxidized intermediates, metals binding (mainly iron and copper), the inhibition of enzymes responsible for the formation of free radicals (oxidase, peroxidase), the activation of antioxidant enzymes (catalase, superoxide dismutase) and the prevention of oxidation of other antioxidants (ascorbic acid, vitamin E) [17].

Table 1: Total phenolic content of polyherbal leaves extract

Concentration (mg\ml)	Aqueous extract (mg\g)	Methanolic extract (mg\g)	LSD value
2.5	3.96 ± 0.07	12 .98 ± 0.11	0.221 **
5	8.17 ± 0.06	24.88 ± 0.23	0.679 **
10	15.52 ± 0.24	46.97 ± 0.07	0.692 **
LSD value	0.498 **	0.513 **	---
** ($P \leq 0.01$).			

Antioxidant activity of polyherbal leaves extracts (DPPH assay)

In this study, the radical scavenging activity of each extract was compared at concentrations of (0.625, 1.25, 2.5, 5 and 10) mg/ml. BHA and vitamin C were used as references. The results showed that the free radical scavenging activity of methanolic extracts was (93.189%) in 10 mg/ml was more effective than aqueous extracts (86.77%) in the same concentration, and It was approach with the natural antioxidant (vitamin C) and artificial antioxidant (BHT) which was (96.40 % and 93.67 %) respectively As shown in Table (2).

Furthermore, the antioxidant activity is expressed as an Effective Concentration (EC_{50}). The half maximal Effective Concentration (EC_{50}) are often refers to the concentration of a drug, toxicant or antibody which induces a response half way between the baseline and maximum after a specified exposure time, it commonly used as a measure of potency of a drug [18].

The radical scavenging capacity (EC_{50}) of methanolic and aqueous extracts were found to be (1 and 1.35 mg/ml) respectively, and the value of BHT and V. C were found to be (0.60 and 0.47 mg/ml) respectively (Figure 1). The effectiveness of the antioxidant properties is inversely correlated with EC_{50} values. Lee

et al., [19] reported that if the EC_{50} value of an extract is less than 10 mg/ml, it indicates that the extract is an effective antioxidant. The EC_{50} value of polyherbal leaves extracts were less than 10 mg /ml, and this indicates that the extracts were an effective antioxidant.

Table 2: Radical scavenging activity of polyherbal leaves extract

Concentration mg\ml	Aqueous extract	Methanolic extract	BHT	Vit. C.	LSD value
0.625	20.86 ±0.16	36.31 ±0.12	60.68 ±0.26	90.23 ±0.19	0.596 **
1.25	51.34 ±0.05	68.52 ±0.17	81.42 ±0.24	91.54 ±0.29	0.698 **
2.5	57.92 ±0.03	80.17 ±0.03	91.28 ±0.04	95.23 ±0.16	0.288 **
5	85.48 ±0.06	92.27 ±0.02	93.13 ±0.01	96.21 ±0.16	0.284 **
10	86.77 ±0.06	93.28 ±0.02	93.67 ±0.01	96.40 ±0.27	0.459 **
LSD value	0.196 **	0.309 **	0.519 **	0.710 **	---

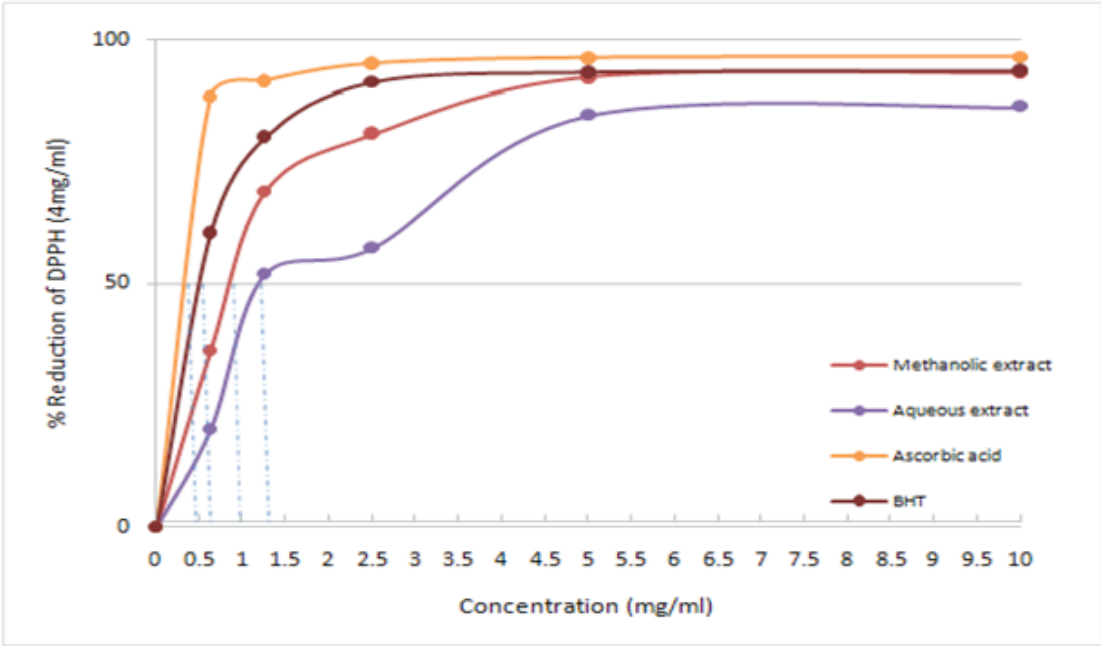

Figure 1: EC_{50} of polyherbal leaves extracts

Acute toxicity test

The study of the acute toxicity of polyherbal leaves extracts shows different signs when treated with different oral doses of aqueous and methanolic extract (Table 3). The experimental mice show appreciable changes in physical activity and shown abnormal responses such as Tacky cardiac, increase breathing, sedation and animal tend to loneliness for one side in different time in dose 2000 mg/kg, but there is no mortality in mice were recorded after 24 hours post treatment. The results showed that the aqueous and methanolic extract of polyherbal leaves practically non toxic according to Hodge and Sterner [20]. Accordingly, to this study, both extracts of polyherbal leaves did not induce lethality in mice when administered orally at doses of began from 100 till reach to 2000 mg/kg. This result suggests that LD_{50} of the extract would be greater than 2000 mg/kg. Therefore, the plant extract can be assumed practically non-toxic.

Table 3: Acute toxicity and mortality rate of polyherbal leaves extracts

Dose of extract mg/kg/ B.W	No. of mice per group	No. of dead / No. of animal	Sign of animal treated with extract
Aqueous extract			
100	6	0/6	Nil
250	6	0/6	Nil
500	6	0/6	Nil
1000	6	0/6	Tacky cardiac, increase breathing, sedation and animal tend to loneliness for one side in different time
2000	6	0/6	Tacky cardiac, increase breathing, sedation and animal tend to loneliness for one side in a different time but it takes a long time than above
Methanolic extract			
100	6	0/6	Nil
250	6	0/6	Nil
500	6	0/6	Interrupted sedation
1000	6	0/6	Tacky cardiac, titanic hair skin, animal tend to loneliness for one side in different time
2000	6	0/6	Tacky cardiac, titanic hair skin, animal tend to loneliness for one side in different time all these signs take a long time than above

Effect of polyherbal leaves extracts on serum glucose level

At the beginning of the experiment, the blood glucose of animals was measured as the (Baseline), after that all animal were injected with alloxan to induce Diabetes (except normal group) and then divided to seven groups and treated with polyherbal leaves extracts and metformin as shown in (Table 4). The results showed that diabetic mice treated with methanolic extract at doses 200 and 400 mg/kg (group 4 and 5) was significant gradual descent decreased ($p < 0.01$) in the serum glucose level after 35 days, which was 113.66 and 107.66 mg/dl respectively when compared with control positive groups 324.00 (group 2). Likewise, the results showed that the serum glucose level decreased ($p < 0.01$) in metformin treatment diabetic mice 100.66 (group 3) compared to control positive groups (group 2), which mean the methanolic extract at doses 400 mg/kg have the same effect of metformin in decrease serum glucose level. Methanolic extracts were more effective than aqueous extracts which was 124.66 and 117.00 mg/dl in concentrations 200 and 400 mg/kg (group 6 and 7) respectively.

Table 4: Effect of polyherbal leaves extracts on serum glucose level

Groups	Baseline	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	LSD Value
Group 1	90.66±1.76	91.33 ±1.20	90.00 ±3.60	95.33 ±0.88	91.33 ±1.20	92.00 ±3.05	91.00 ±2.08	6.63 NS
Group 2	89.33±1.85	299.66±1.20	303.66±2.84	300.33±3.48	308.66±5.60	317.33 ±2.60	324.00±2.65	9.61 **
Group 3	89.33±2.18	300.00±2.51	227.66±1.45	192.00±1.73	140.33±0.33	106.66 ±2.18	100.66±0.88	5.36 **
Group 4	91.66±1.45	302.66±4.33	244.66±2.02	211.00±1.15	152.00±1.73	127.66 ±0.88	113.66±1.45	6.50 **
Group 5	90.33±1.20	300.00±6.65	231.66±2.02	201.00±1.15	145.00±1.15	120.33±1.2 0	107.66±2.60	8.93 **
Group 6	88.33±1.20	296.33±5.23	257.66±0.88	228.66±0.88	165.33±0.88	139.66 ±2.60	124.66±0.33	7.07 **
Group 7	88.00±1.52	292.66±3.48	249.33±1.45	217.00±1.15	155.66±2.02	131.66 ±1.45	117.00±2.31	6.22 **
LSD Value	4.95 NS	12.12 **	6.71 **	5.21 **	7.44 **	6.48 **	5.89 **	---
** (P≤0.01).								

Group 1: Control,
Group 2: Alloxan (150 mg/kg),
Group 3: Diabetic mice + Metformin (150 mg/kg),
Group 4: Diabetic mice + Polyherbal methanolic extract (200 mg/kg),
Group 5: Diabetic mice + Polyherbal methanolic extract (400 mg/kg),
Group 6: Diabetic mice + Polyherbal aqueous extract (200 mg/kg),
Group 7: Diabetic mice + Polyherbal aqueous extract (400 mg/kg).

In the present study, oral administration of polyherbal formulation at dose levels of 200 and 400mg/kg for 35 days enhances insulin production. This may be due to the regenerating effect of pancreatic β-Cells. The polyherbal formulation increased insulin levels in a dose dependent manner and was comparable with that of standard drug [21]. Antidiabetic potential of phytochemicals as Alkaloids produce antihyperglycaemic action by potentiating pancreatic secretion of insulin from β-cell of islets or by enhancing transport of blood glucose to peripheral tissue [22]. According to Ayurveda, there are several medicinal plants that have been identified to possess antidiabetic potential. Most of the herbal preparations from these medicinal plants are reported to have minimal or no side effects [23]. Due to the antioxidant properties of polyphenols, these compounds can play an important role in antidiabetic prevention and therapy [24]. Flavonoids might prove to be important for alternative diabetic treatment, as it helps in preventing β-cell apoptosis, promoting β-cell proliferation and insulin secretion, and enhancing insulin activity [25]. Triterpenoid and steroidal glycosides are collectively referred to as saponins; these compounds are known to possess potent hypoglycaemic activity [26]. The phenolic compounds may exhibit their hypoglycaemic activities by increasing the levels of serum insulin, increasing the sensitivity of tissues to insulin action, stimulating the activity of enzymes of glucose utilization and inhibiting the activity of α-amylase [27]. Tannins also play an important role in preventing diabetic complications by reducing the formation of advanced glycation end products and oxidative stress [28].

Effect of polyherbal leaves extracts on body weight

At present, the treatment of diabetes mainly involves a sustained reduction in hyperglycaemia by the use of hypoglycaemic drugs in addition to insulin. More so, myriads of medicinal plants seem to reveal potential hypoglycaemic activity and antioxidant action with desirable properties [29]. The present study indicated that the final body weight of positive control (diabetes induction) was significantly decreased (23.97 gram) when compared with control negative group 30.26 gram (group 1). Otherwise, the reduction in body weight was partially restored or improved upon administration of

polyherbal methanolic and aqueous extracts at doses 200 and 400 mg/kg (groups 4, 5, 6, 7) when compared with control positive group as shown in Table (5). Daye *et al*. [30] reported that glibenclamide suppressed the decrease in the body weight, while the suppression of weight loss in this study was achieved using polyherbal extracts. Chinwe *et al*. [31] suggest that the *Garcinia kola* extract has shown to be a potential agent for the treatment of diabetes mellitus and restoration of body weight loss in alloxan induced diabetic rats. Furthermore, Shahadat *et al*. [32] revealed that the final body weights of different treatments with plant extracts have showed significantly increased from the initial body weight.

Table 5: Effect of polyherbal leaves extracts on body weight

Groups	Initial weight (g)	Final weight (g) After 5 weeks
Group 1	27.30 ±0.21	30.26 ±0.18
Group 2	31.30 ±0.47	23.97 ±0.23
Group 3	30.76 ±0.23	30.13 ±0.20
Group 4	29.53 ±0.08	28.60 ±0.05
Group 5	31.30 ±0.37	30.53 ±0.35
Group 6	29.73 ±0.08	28.26 ±0.12
Group 7	28.66 ±0.14	27.53 ±0.12
LSD value	0.811 **	0.614 **
** ($P \leq 0.01$).		

Group 1: Control,
Group 2: Alloxan (150 mg/kg),
Group 3: Diabetic mice + Metformin (150 mg/kg),
Group 4: Diabetic mice + Polyherbal methanolic extract (200 mg/kg),
Group 5: Diabetic mice + Polyherbal methanolic extract (400 mg/kg),
Group 6: Diabetic mice + Polyherbal aqueous extract (200 mg/kg),
Group 7: Diabetic mice + Polyherbal aqueous extract (400 mg/kg).

Conclusion

Polyherbal methanolic extract contained relatively a higher amount of phenolic compounds and also exhibited a superior antioxidant activity, even comparable with the synthetic antioxidants (BHA). Furthermore, the extracts of polyherbal have antidiabetic effect on diabetic mice induced by alloxan, therefore could be taken as a dietary supplement as an anti-hyperglycaemic.

Conflict of Interest: The authors declared that present study was performed in absence of any conflict of interest.

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