Int J Pharm Bio Sci 2015 July; 6(3): (B) 198 - 214

Research Article **Biochemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

JATROPHA CURCAS REPAIRING EFFECT ON ADHESION MOLECULES, DNA DAMAGE AND GENE EXPRESSION ALTERATION IN STZ-INDUCED DIABETIC RATS

FAROUK K. EL-BAZ *[1], HANAN F. ALY[2], WAGDY K. B. KHALIL[3], HODA F. BOOLES[3] AND SAFAA A. SAAD[1]

[1]*Plant Biochemistry Department, National Research Centre (NRC), 33 EL Bohouth st. (former EL Tahrir st.), Dokki, Giza, Egypt, P.O.12622.*
[2]*Therapeutic Chemistry Department, National Research Centre (NRC), 33 EL Bohouth st. (former EL Tahrir st.), Dokki, Giza, Egypt, P.O.12622.*
[3] *Cell Biology Department, National Research Centre (NRC), 33 EL Bohouth st. (former EL Tahrir st.), Dokki, Giza, Egypt, P.O.12622.*

ABSTRACT

The recent research converges on the evaluation of *Jatropha curcas* different extracts on some biochemical parameters in streptozotocin (STZ)-induced diabetic rats. Diabetes mellitus (DM) was induced using STZ (45 mg/kg b.w) while, *J. curcas* extracts were orally administered at a dose of 250mg/kg/day and glibenclamide (antidiabetic drug) at a dose of 10mg/kg/day for 30 days. Adhesion molecules; VCAM-1 and ICAM-1 were measured in blood serum of rats and the results showed that VCAM-1 and ICAM-1 levels were significantly increased in diabetic rats as compared to controls. The results showed that *J. curcas* extracts have reduced VCAM-1 and ICAM-1 high levels in diabetic rats. DNA fragmentation was determined using diphenylamine reaction as well as DNA gel electrophoresis laddering assays and the expression of diabetes related genes was determined by qRT-PCR. The results revealed that the administration of *J. curcas* extracts exhibited relatively the rates of DNA damage similar to those in the control group. Moreover, *J. curcas* extracts altered the expression of diabetes related genes and appeared similar to those in control group. Thus, *J. curcas* extracts might be a promising regulator supplement for hyperglycemia and the expression of genes related DM.

KEYWORDS: ***Jatropha curcas*; streptozotocin; adhesion molecules; DNA damage; diabetes related genes.**

*Corresponding author

FAROUK K. EL-BAZ
Plant Biochemistry Department, National Research Centre (NRC),
33 EL Bohouth st. (former EL Tahrir st.), Dokki, Giza, Egypt, P.O.12622.

INTRODUCTION

Adhesion molecules are important in cell-cell and cell-basement membrane interactions. Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) play a role in cell adhesion to the vascular endothelium. Type 2 diabetes mellitus (T2DM) is powerful and independent risk factors for coronary artery disease, stroke, and peripheral arterial disease. Accelerated atherosclerosis is the morbidity and mortality in this common metabolic disorder [1]. It was proved that, hyperglycemia in diabetic patients can trigger endothelial damage through increased oxidative stress and up-regulation of cellular adhesion molecules [2]. On the other hand, the increased production of reactive oxygen species (ROS) plays a key role in pathogenesis of DM complications where, ROS are generated by exogenous and endogenous factors such as during hyperglycemia ensued by oxidative stress [3]. The authors added that, oxidative stress induces DNA damage and when DNA damage exceeds the cellular capacity to repair it, the accumulation of errors can overwhelm the cell resulting in cell death or fixation of genome mutations that can be transmitted to future cell generations. Additionally, mutations can promote genome instability and directly lead to various human diseases such as cancer, neurological abnormalities, immunodeficiency, and premature aging [4,5]. Oxidative stress induced inflammatory responses cause damage to the vasculature and may play an important role in the development of many diseases. Activation of endothelial cells by oxidants may lead to a wide range of functional changes such as an increased expression of VCAM-1 and ICAM-1[6]. In addition, ROS can cause strand breaks in DNA and base modifications, including oxidation of guanine residues to 8-hydroxy-2'deoxyguanosine (8-OHdG) - an oxidized nucleoside of DNA that is the most frequently detected and studied DNA lesion [7]. Medicinal plants have been used to cure or prevent diseases leading to the promotion of good health. The benefit of these plants is related to the secondary metabolites that are produced by the plants, even though plants produced these secondary metabolites for the benefits of the plant itself as defense against infection and injury, but it was found that the secondary metabolites have benefits to the human health and curing human diseases [8]. Secondary metabolites of medicinal plants produce a natural source of antioxidants. These natural antioxidants gained major attention and importance towards treatment of various free radical-related diseases such as cancer, asthma, atherosclerosis, arthritis, aging, and autoimmune disorders, several stress related diseases, including cataracts, cognitive dysfunction, myocardial infarction, and diabetes, and several cardiovascular and neurodegenerative diseases [9,10]. *J. curcas* (Euphorbiaceae family) is a shrub or small tree that gained its name from the Greek word *jatro´s* (doctor) and *trophe´* (food), which connotes its medicinal uses. Several biological activities were reported to the plant throughout the amelioration of neuropathic pain and treating inflammatory diseases [11]. Different parts of the plant, including the leaves, fruits, latex and bark contain bioactive compounds such as glycosides, tannins, phytosterol, flavonoids and steroidal sapogenins with several medicinal properties [12,13]. So, the present research aims to evaluate the ameliorative effects of *J. curcas* extracts on increased levels of adhesion molecules as well as the DNA damage attributed to DM deadly disease.

MATERIALS AND METHODS

(i) Chemicals and reagents

All chemicals in the present study are of analytical grade, products of Sigma, Merck and Aldrich. All kits were the products of Biosystems (Alcobendas, Madrid, Spain), Sigma Chemical Company (St. Louis, MO, USA), Biodiagnostic Company (Cairo, Egypt).

This article can be downloaded from www.ijpbs.net

(ii) Collection and preparation of plant material

J. curcas fresh leaves were collected from the farm of Aromatic and Medicinal Plant Department, Agriculture Research Centre, Egypt. The plant was kindly authenticated by Mrs Treas Labib, Herbarium section, El-Orman Botanical Garden, Giza, Egypt. The leaves were washed with tap water, then with distilled water to remove dust and dirt. Leaves were air dried under shade, then grinded and homogenized to coarse powder finally stored in opaque screw tight jars until use.

(iii) Successive and crude extracts preparation

About 2.5 kg powdered leaves were extracted successively by a cold maceration method with different solvents of increasing polarity on shaker (Heidolph) *i.e.* petroleum ether, ethyl acetate and methanol. The marc was dried each time before extraction with next solvent. After complete extraction the extracts were filtered by using Whatman grade No. 4 filter paper and Buchner. Filtrates were concentrated using Rotary evaporator (Heidolph) at 40°C under vacuum and stored in refrigerator (4°C) till biological assay and chemical analysis. While, the crude methanolic extract was prepared by cold maceration in methanol of 300 g of *J. curcas* powdered leaves on shaker. The extract was filtered by using Whatman grade No. 4 filter paper and Buchner. The filtrate was concentrated using Rotary evaporator at 40°C under vacuum and stored in refrigerator (4°C) till biological assay and chemical analysis.

(iv) Biological experiment:

1. Animals

Female albino rats (n=110) weighted (150-200 g), were used for the evaluation of anti-diabetic effects of *J. curcas* extracts and provided by the Animal House of the National Research Centre (NRC) and housed in a temperature-controlled environment (26-29°C) with a fixed light/dark cycle for one week as an adaptation period to acclimatize under normal combination with free access to water and food. The present study is approved by the Ethical Committee of the NRC, Egypt, provided that the animals will not suffer at any stage of the experiment.

2. Experimental design[14-17]

One hundred and ten rats were selected for this study and divided into eleven groups of ten rats each as follows:

Group 1: Normal, healthy control rats, Groups 2-5: Normal rats treated orally with 250 mg/kg body of petroleum ether, ethyl acetate, successive and crude methanolic extracts for 30 days. Group 6: Is considered as diabetic group; where type2 diabetes was induced by intraperitoneally injection of a single dose of STZ (45 mg/kg body weight) dissolved in 0.01 M citrate buffer immediately before use. After injection, animals had free access to food, water and were given 5% glucose solution to drink overnight to encounter hypoglycaemic shock. Animals were checked daily for the presence of glycosuria. Animals were considered to be diabetic if glycosuria was present for 3 consecutive days. After 3 days of STZ injection fasting blood samples were obtained and blood sugar was determined (≥300 mg/dl). Hyperglycemic rats were used for the experiment and classified as follows:

Groups 7-10: Diabetic rats oral administered 250 mg/kg body weight petroleum ether, ethyl acetate, successive and crude methanolic extracts for 30 days respectively, Groups 11: Diabetic rats administered orally antidiabetic glibenclamide reference drug 10 mg/kg body weight daily for 30 days.

3. Sample preparations

After 30 days of treatments, rats were fasted overnight (12-14 hours), anesthetized by diethyl ether and blood collected by puncture of the sublingual vein in clean and dry test tube, left 10 minutes to clot and centrifuged at 3000 r.p.m for serum separation. The separated serum was used for biochemical analysis of adhesion molecules (VCAM-1 and ICAM-1).

4. Biochemical examination

Estimation of serum adhesion molecules; VCAM-1 and ICAM-1 was performed by ELISA; a sandwich enzyme immunoassay.

Calculation:

$$\% \text{ change} = \frac{\text{Mean of control} - \text{mean of treated} \times 100}{\text{Mean of control}}$$

$$\% \text{ of improvement} = \frac{\text{Mean of treated} - \text{mean of disease} \times 100}{\text{Mean of control}}$$

5. DNA Fragmentation Analysis

a- Diphenylamine reaction procedure [18]

Rats liver and heart tissues were used to determine the quantitative profile of the DNA fragmentation. Liver and heart samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml of lysis buffer containing, 10 mM tris-HCl (pH 8), 1 mM EDTA, 0.2% triton X-100, centrifuged at 10 000 rpm (Eppendorf) for 20 min at 4°C. The pellets were re-suspended in 0.5 ml of lysis buffer. To the pellets (P) and the supernatants (S), 0.5 ml of 25% tri-chloroacetic acid (TCA) was added and incubated at 4°C for 24 h. The samples were then centrifuged for 20 min at 10 000 rpm (Eppendorf) at 4°C and the pellets were suspended in 80 ml of 5% TCA, followed by incubation at 83°C for 20 min. Subsequently, to each sample 160 ml of DPA solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg:ml)] was added and incubated at room temperature for 24 h. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm wavelength using the formula:

$$\% \text{ Fragmented DNA} = \frac{OD(S)}{OD(S) + OD(P)} \times 100$$

b- DNA gel Electrophoresis Laddering Assay [19]

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA. Briefly, liver tissues were homogenized, washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris–HCl, 10 mM EDTA. 0.5% Triton, and 100 µg/ml proteinase K, pH 8.0) for overnight at 37 °C. The lysate was then incubated with 100 µg/ml DNase-free RNase for 2h at 37 °C, followed by three extractions of an equal volume of phenol/chloroform (1:1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 °C. The extracted DNA was precipitated in 2 volume of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at -20 °C for 1h, followed by centrifuging at 15,000 rpm for 15 min at 4 °C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris–HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris–acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

6. Expression of diabetes related genes by qRT-PCR

a- Isolation of total RNA

Total RNA was isolated from liver and heart tissues of male rats by the standard TRIzol® Reagent extraction method (Invitrogen, Germany). Briefly, tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. Afterwards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then the samples were vortexed vigorously for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged for no more than 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a

colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 °C for 10 minutes and centrifuged at not more than 12,000 x g for 10 minutes at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes at 4 °C. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

b- Reverse transcription (RT) reaction

The complete Poly(A)$^+$ RNA isolated from male rat liver and heart tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (qRT-PCR).

c- Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers**.** Each experiment included a distilled water control. the specific gene primer sequences and PCR cycling conditions are listed in (Table 1). The quantitative values of RT-PCR (qRT-PCR) of diabetes related genes (Insulin-like growth factor 1 receptor, IGF1R, Insulin-like growth factor binding protein 2, IGFBP2, Insulin-like growth factor binding protein 3, IGFBP3, Glutamate receptor, ionotropic, N-methyl D-aspartate 2C, GRIN2C and Vascular endothelial growth factor A,VEGFA) were normalized on the bases of ß-actin expression. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Table 1
Oligonucleotide primers used for the analysis of diabetes related genes by qRT-PCR

Gene name	Primer Sequence (5′-3′)	Reference/ or Accession number	Annealing temp (ºC)
β-actin	F: GTG GGC CGC TCT AGG CAC CAA	*(Khalil and Booles)* [20]	64.5
	R: CTC TTT GAT GTC ACG CAC GAT TTC		
Insulin-like growth factor 1 receptor (IGF1R)	F: CCA ACA AGT TCG TCC ACA G	NM_052807	59
	R: AGT CCG TCT CGT AGA TGT C		
Insulin-like growth factor binding protein 2 (IGFBP2)	F: GGG TCC TCT GGA ACA TCT C	NM_013122	59
	R: GTC CAT TCA GAG ACA TCT TGC		
Insulin-like growth factor binding protein 3 (IGFBP3)	F: ACA GAC ACC CAG AAC TTC TC	NM_012588	59
	R: CAG CAC ATT GAG GAA CTT CAG		
Glutamate receptor, ionotropic, N-methyl D-aspartate 2C (GRIN2C)	F: GGC CCA GCT TTT GAC CTT AGT	U08259	59
	R: CCT GTG ACC ACC GCA AGA G		
Vascular endothelial growth factor A (VEGFA)	F: AGG AAA GGG AAA GGG TCA	NM_031836	57
	R: ACA AAT GCT TTC TCC GCT		

F: Forward primers; R: Reverse primers

d- Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet:

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method if Ef for the target (IGF1R, IGFBP2, IGFBP3, GRIN2C and VEGFA) and the reference primers (β-Actin) as follows:

$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(reference,\ test)}$,

$\Delta CT(calibrator) = C_{T(target,\ calibrator)} - C_{T(reference,\ calibrator)}$,

$\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}$.

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

7. Statistical analysis

All data were subjected to one-way analysis of variance ANOVA and the significance of the differences between means was tested using co-state computer program and Tukey's HSD (Honestly Significant Difference) test ($P < 0.05$). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard error.

RESULTS

1. *Effect of successive extracts as well as crude methanolic extract of J. curcas on adhesion molecules*

The effect of different extracts of *J. curcas* on adhesion molecules in STZ-induced diabetic rats and different therapeutic groups was showed in Table 2. The results demonstrated insignificant change in VCAM-1 and ICAM-1 levels with different extracts supplemented to normal rats as compared to untreated control one. Diabetic rats showed significant increase in both VCAM-1 and ICAM-1 levels with percentages 57.34 and 13.16%, respectively. VCAM-1 levels were markedly ameliorated in diabetic-treated groups with petroleum ether, ethyl acetate,

successive methanolic, crude methanolic extracts and glibenclamide as compared to untreated one with percentages of improvement 30.99, 34.12, 35.79, 38.65 and 38.36%, respectively. On the other hand, all extracts showed insignificant change in ICAM-1 levels. As shown in Table (2), the highest reduction in VCAM-1 and ICAM levels was obtained with crude methanolic extract followed by successivemethanolicextract and petroleum ether

Effect of Jatropha curcas extracts on rates of DNA fragmentation

The results of the DNA fragmentation assay revealed that treatment of diabetic rats with different extracts of *J. curcas* induced different rats of DNA fragmentation (Figure 1 and Table 3).The rate of DNA fragmentation in liver and heart tissues of control rats was observed at low rate of DNA damage (Table 3). However, diabetic-female rats induced high rates of DNA fragmentation which were 34.6 and 31.2 in liver and heart tissues, compared with 9.8 and 9.6 in control rats, respectively. The different extracts of *J. curcas* exhibited low rates of DNA damage either in liver or heart tissues of non-diabetic rats, whereas the rate of DNA damage in groups treated with petroleum ether (PE), ethyl acetate (EA) and successive methanolic (SM) extracts were relatively similar to those in control group (Table 3). However, treatment of female rats with crude methanolic (CM) extract exhibited more DNA damage than the other extracts. On the other hand, treatment of diabetic rats with PEE, EAE and SME extracts revealed significantly low rates of DNA damage compared to DM rats. Moreover, treatment of female rats with CME decreased significantly the rate of DNA damage induced in DM rats, however, this decrease in the rate of DNA damage was higher than those induced by other extracts.

Table 2

Effects of successive extracts and crude methanolic extract of J. curcas supplementation on adhesion molecules; VCAM-1 and ICAM-1 levels in normal, STZ-induced diabetic rats and different therapeutic groups

Groups	Parameters	VCAM-1 (ng/ml)	ICAM-1 (ng/ml)
Normal rats	Mean± S.D.	12118.92±7.73^{e}	254.46±2.08^{c}
Normal rats treated with petroleum ether extract (PEE)	Mean ±S.D % Change to control	13212.25±179.51^{e} 9.02	247.13±1.79^{c} 2.88
Normal rats treated with e ethyl acetate extract (EAE)	Mean ±S.D % Change to control	13285.06±67.36^{e} 9.62	247.20±1.70^{c} 2.85
Normal rats treated with successive methanolic extract (SME)	Mean ±S.D. % Change to control	12978.48±265.16^{e} 7.09	245.65±3.30^{c} 3.46
Normal rats treated with crude methanolic extract (CME)	Mean ±S.D. % Change to control	13191.25±6.68^{e} 8.84	248.63±4.03^{c} 2.29
Diabetic rats (DM)	Mean ±S.D. % Change to control	19068.33±280.57^{a} 57.34	287.96±1.96^{a} 13.16
Diabetic rats treated with petroleum ether extract (DM+ PEE)	Mean ±S.D. % Change to control % Of improvement	15312.62±473.47^{b} 26.35 30.99	260.25±4.44^{bc} 2.27 10.89
Diabetic rats treated with ethyl acetate extract (DM+ EAE)	Mean ±S.D. % Change to control % Of improvement	14933.16±104.78^{bc} 23.22 34.12	255.12±1.34^{bc} 0.26 12.90
Diabetic rats treated with successive methanolic extract (DM+ SME)	Mean ±S.D. % Change to control % Of improvement	14730±464.11^{c} 21.54 35.79	257.42±0.54^{bc} 1.16 12.00
Diabetic rats treated with crude methanolic extract (DM+ CME)	Mean ±S.D. % Change to control % Of improvement	14383.35±125.50^{c} 18.68 38.65	248.13±1.45^{c} 2.48 15.65
Diabetic rats treated with antidiabetic drug (DM+ Glibenclamide)	Mean ±S.D. % Change to control % Of improvement	14419.19±511.76^{c} 18.98 38.36	246.55±2.53^{c} 3.11 16.27

All values are means±SD of 10 rats in each group

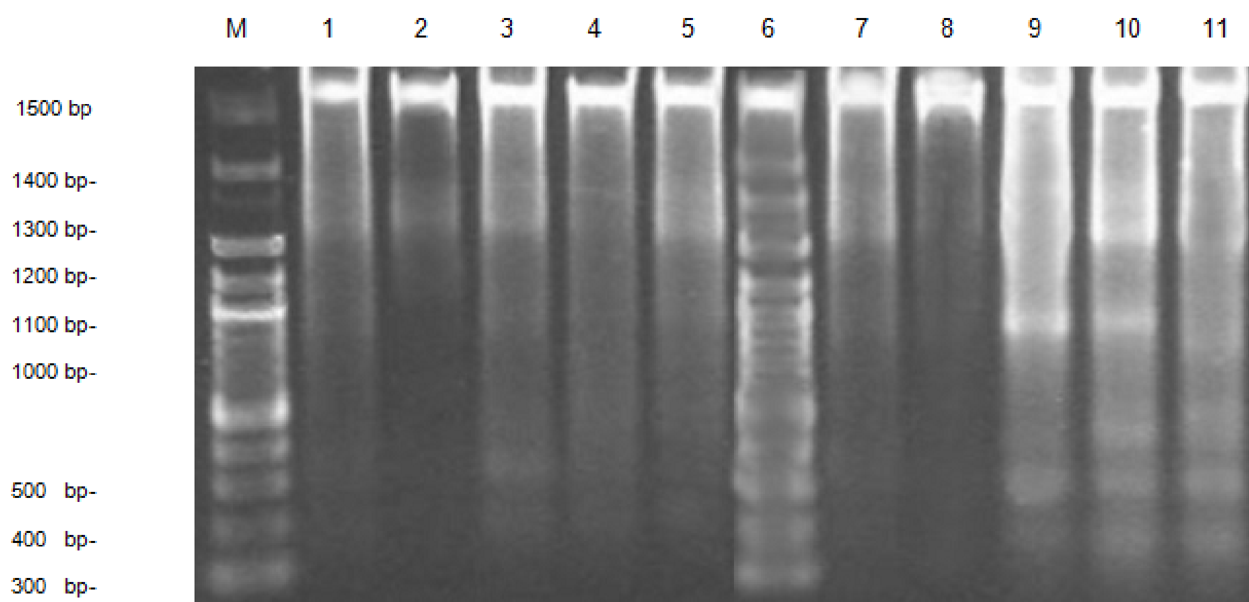
-Data were analyzed using analysis of variance (ANOVA) combined with co –state computer program, where unshared letter is significant at p≤0.05

Table 3
DNA fragmentation in liver and heart tissues of male DM-rats treated with several extracts of Jatropha curcas analyzed by diphenylamine reaction procedure.

Treatment	DNA Fragmentation (%)	
	Liver (Mean±SEM)	Heart (Mean±SEM)
Control	09.8±0.6[e]	09.6±0.5[e]
PEE	10.2±1.4[e]	10.1±1.2[e]
EAE	09.7±1.2[e]	09.4±1.1[e]
SME	11.6±1.1[de]	11.2±1.3[de]
CME	13.2±1.3[d]	12.8±1.2[d]
DM	34.6±3.7[a]	31.2±3.1[a]
DM+ PEE	18.3±1.5[c]	17.7±1.4[c]
DM+ EAE	17.4±1.6[c]	16.3±1.3[c]
DM+ SME	21.8±2.1[bc]	20.2±1.7[bc]
DM+ CME	23.6±2.3[b]	22.3±2.1[b]
DM+ Glibenclamide	19.1±1.3[c]	18.2±1.3[c]

PEE: Petroleum ether extract; EAE: Ethyl acetate extract; SME: Successive methanolic extract; CME: Crude methanolic extract; DM: Diabetes mellitus

Figure 1
DNA fragmentation in liver tissues of male DM-rats treated with several extracts of Jatropha curcas. M: DNA marker. Lane 1 represents PCR products of untreated control samples; Lanes 2-5 represent rats treated with PEE, EAE, SME and CME extracts, respectively; lane 6 represents DM-rats; Lanes 7-10 represent DM-rats treated with PEE, EAE, SME and CME extracts, respectively; lane 6 represents DM-rats treated with glibenclamide drug.



3. *Effect of J. curcas extracts on the expression of diabetes related genes (IGF1R, IGFBP2, IGFBP3, GRIN2C and VEGFA)*

The expression of diabetes-associated genes, in the STZ-induced diabetic rats treated with different extracts of *J. curcas* was determined using qRT-PCR (Figures 2-6). The results

revealed that diabetic rats showed significantly low expression values of IGF1R, IGFBP2 and IGFBP3 (in liver) and VEGFA (in heart) comparing with the control rats (Figures 2, 3, 4 & 6). However, the expression of GRIN2C gene was increased significantly in heart tissues of diabetic rats as compared to control group (Figure 5). On the other hand, treatment of non-diabetic rats with PE; EA, SM and CM extracts exhibited expression values of IGF1R, IGFBP2, IGFBP3, GRIN2C and VEGFA genes relatively similar to those in control rats (Figures 2-6). Moreover, treatment of diabetic rats with PE; EA, SM and CM extracts exhibited significantly higher expression values of IGF1R, IGFBP2, IGFBP3 (in liver) and VEGFA (in heart) and lower expression of GRIN2C gene (in heart) as compared to those in diabetic rats (Figures 2-6). Furthermore, PE and EA extracts exhibited more effective impact on the expression of IGF1R, IGFBP2, IGFBP3, GRIN2C and VEGFA genes as compared to other extracts.

Figure 2

The alterations of IGF1R mRNA in liver tissues isolated from male DM-rats treated with several extracts of Jatropha curcas. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different (P<0.05).

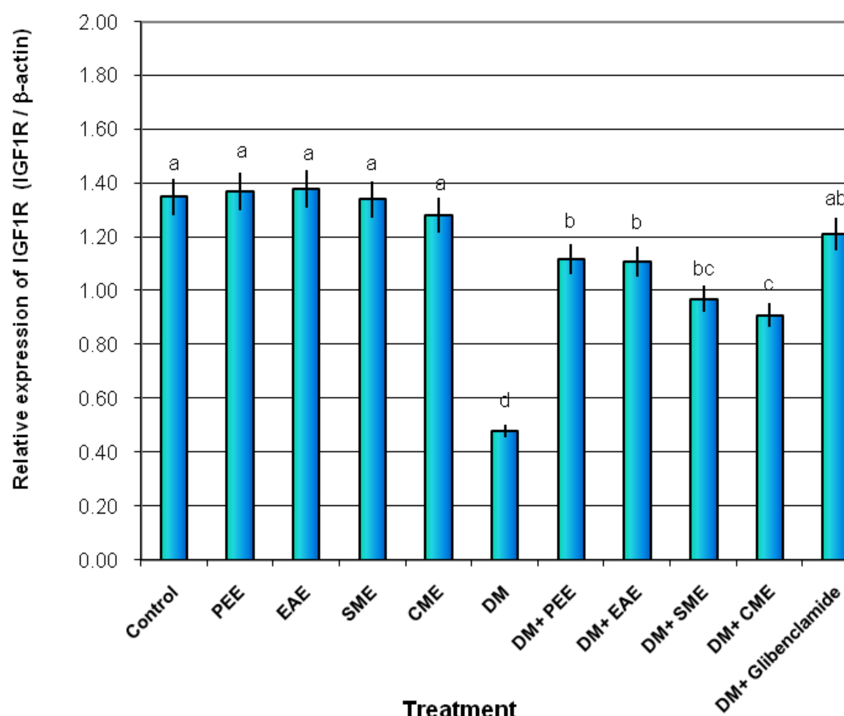


Figure 3

The alterations of IGFBP2 mRNA in liver tissues isolated from male DM-rats treated with several extracts of Jatropha curcas. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different (P<0.05).

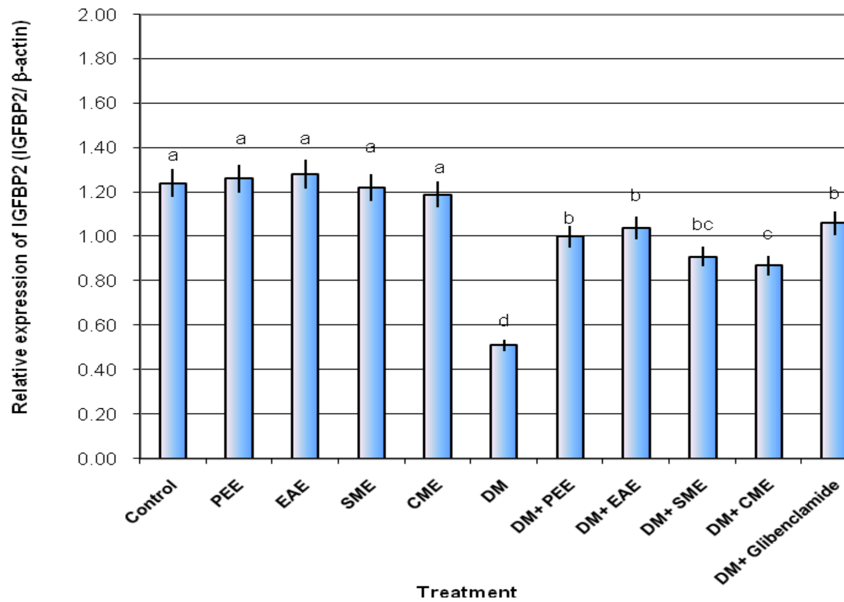


Figure 4

The alterations of IGFBP3 mRNA in liver tissues isolated from male DM-rats treated with several extracts of Jatropha curcas. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different (P<0.05).

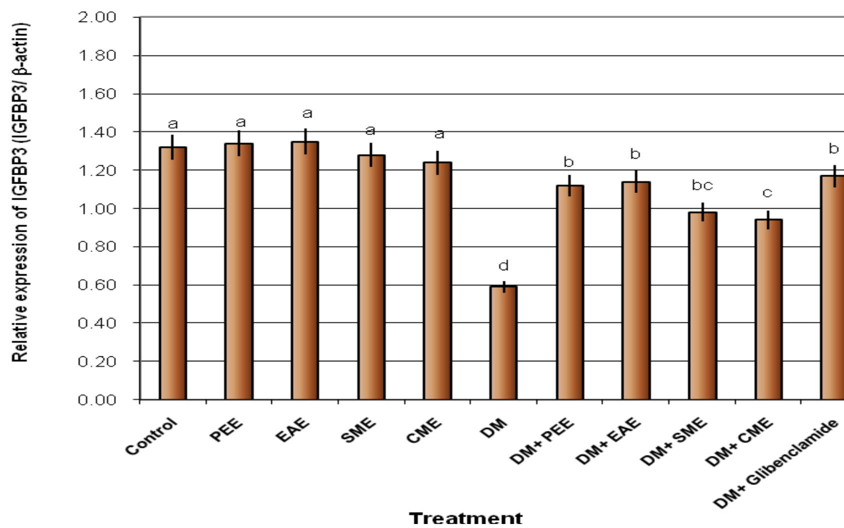


Figure 5

The alterations of GRIN2C mRNA in heart tissues isolated from male DM-rats treated with several extracts of Jatropha curcas. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different (P<0.05).

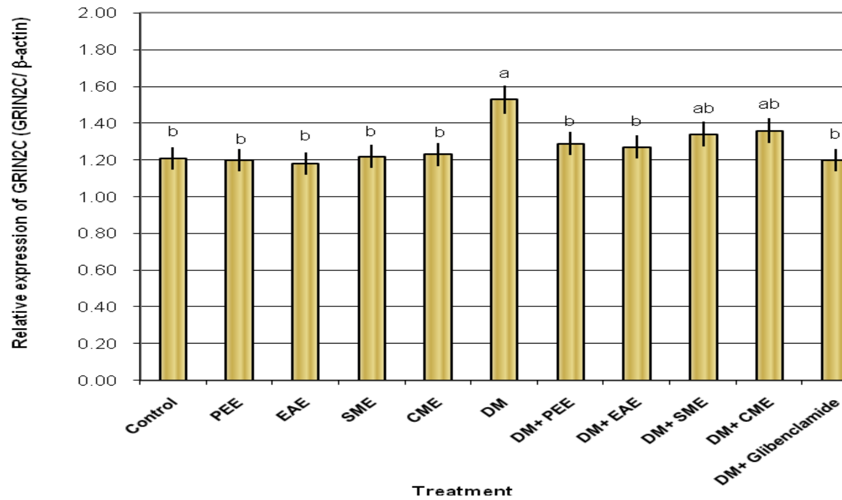
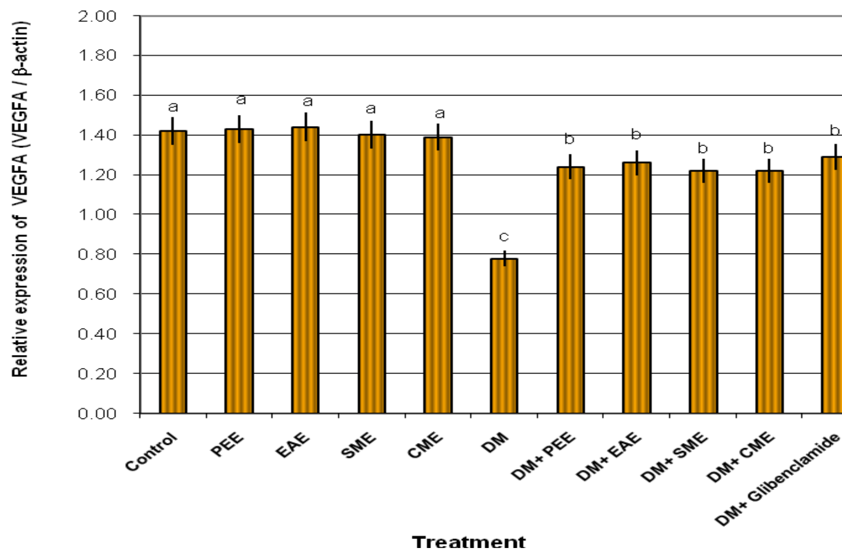


Figure 6

The alterations of VEGFA mRNA in heart tissues isolated from male DM-rats treated with several extracts of Jatropha curcas. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different (P<0.05).



DISCUSSION

A single cell layer lining the vascular wall, called the vascular endothelium, plays an important role in maintaining the structure and function of vessels. Besides, being the vascular endothelium a mechanical barrier between blood and vessel wall, it is also the origin of production for different bioactive factors that regulate vascular tone, coagulation, cell proliferation, cell death, and inflammation [21].The cytokines can be represented in two shapes including the anti-inflammatory markers such as IL-1β, IL-6 and TNF-α, the second shape is the adhesion molecules such as ICAM-1 and VCAM-1 [22]. ICAM-1 and VCAM-1 belong to the Ig super family [23]. Where, ICAM-1 is widely distributed on leucocytes, endothelial cells, fibroblasts and epithelial cells and VCAM-1 is expressed on monocytes, endothelial cells and synovial cells [24,25]. Large evidence has shown strong associations of circulating levels of endothelial adhesion molecules with insulin resistance in non-diabetic individuals or with type 2 diabetic patients [26]. Besides, the concept that insulin resistance might be related to endothelial dysfunction [27]. This is may be due to insulin enhances nitric oxide production within the endothelial cells and nitric oxide modulates a number of endothelial functions, including the expression of adhesion molecules [28]. In a good connection with the present results, in type 2 diabetic patients there is an increase risk for developing infectious diseases and sepsis, and it may lead to mortality because of the ratio of infections can reach nearly twice when compared to non-diabetic patients [29]. The elevated levels of adhesion molecules in diabetic condition may be explained on the basis of, activation of macrophages can produce many of the cytokines known to stimulate endothelial VCAM-1 expression, the matter leading to macrophage accumulation and this consequently may leads to VCAM-1 expression [30]. In parallel results, ICAM-1 is one of the most important intercellular adhesion molecules involved in atherogenesis and noticed increased circulating ICAM-1 plasma levels in NIDDM patients that may be resulted from the acute increase of plasma glucose which leads to produce an oxidative stress and induce cellular expression of ICAM-1 [31]. In agreement with the present results, a significant increase in the immune histochemical expression of ICAM-1 and VCAM-1 proteins in diabetic patients that can reflect the inflammatory nature of this state and suggested a possible role for these adhesion molecules in the pathogenesis of diabetic micro angiopathy [32]. The present results clearly demonstrate amelioration with successive extracts as well as crude methanolic extract of *J. curcas* treatment to diabetic rats. To interpret the suppressing effect of plant extracts on adhesion molecules, the attenuation activity of VCAM-1 and ICAM-1 expression, and this effect was mediated by partial blockage of nuclear factor-kappa B (NF-κB) activation [33]. The antioxidant compounds have an inhibiting activity towards TNF-α-stimulated VCAM-1 and ICAM-1expression *via* a mechanism involving NF-κB and activator protein-1 (AP-1) [34,35]. Methanolic extract of *J. curcas* leaves contained bioactive compounds including, steroids, phenolics and flavones [36]. Flavonoids can support health by strengthening capillaries and other connective tissue and some function as anti-inflammatory, antihistaminic and antiviral agents [37]. Endothelial dysfunction whether acute or chronic, can be counteracted by the administration of phenolic compounds [38]. So, it is probable that the enhancement of endothelial dysfunction effect of *J. curcas* extracts may be referred to its flavonoids and phenolics content. There is a correlation between diabetes and oxidative tissue damage arising from free radical release [39]. Diabetes causes increased free radical release which results in the alterations of liver tissue superoxide dismutase (SOD) and catalase (CAT) [39]. The administration of plant extract with antioxidant potentials was meant to create a balance between free radical release and free radical elimination [39]. Antioxidants are closely related to their bio-functionalities, such as the reduction of cellular abnormalities like DNA damage, mutagenesis, carcinogenesis and which is also associated with free radical

propagation in biological systems [40]. This study showed that different extracts of *J. curcas* were capable of scavenging hydroxyl and may have a stronger hydroxyl radical scavenging activity. The present study revealed that *J. curcas* extracts especially PE and EA extracts decreased significantly the rate of the DNA damage in diabetic rats induced by STZ treatment. The current results indicated that both the extracts of *J. curcas* had a prominent effect on hydroxyl radical/and or super oxide scavenging. These results were in agreement with those of Sundari et al. [41]. The authors reported that, *J. curcas* fractions decreased the DNA damage in human peripheral blood lymphocytes exposed to UVB-irradiation. In addition, the recent study revealed that detoxified *J. curcas* seed meal decreased the DNA fragmentation induced by benzene exposure in male rats [42]. Polyphenolic compounds present in these plant parts have been associated with anti-diabetic, antihypertensive effects and favorable to cardiovascular health [43]. Moreover, flavonoids, coumarins and other polyphenols present in the leaf, stem bark and root extracts of *J.curcas* are well known for their strong antioxidant properties which play important roles in protecting cells and organs from oxidative damage [44]. The free radical scavenging activity of the *J. curcas* fractions might be due to the presence of steroids and terpenoids as they are known to occur in *J. curcas* plant [45]. Previous report showed that high concentrations of the methanolic extract have been reported to be more effective in quenching free radicals in the system [46]. The free radical; 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical that has been used extensively to determine the free radical scavenging ability of various compounds, as a measure of their antioxidant potential [47]. The present study suggested that *J. curcas* extracts possessed a quantitative examination and showed that there was a general trend of significantly higher antioxidant activity. It has been well established that plant extracts and their active compounds enhances DNA repair mechanism and inhibits DNA strand breaks in radiation exposed cells [48]. Antioxidant potential of *J. curcas* has been proposed for its anti-genotoxic potential. Because DNA damage induced by benzene is mainly mediated by ROS, compound with antioxidant potential has capable to intercept the ROS before it attack DNA. Several investigators have demonstrated that natural antioxidants scavenge ROS and protect cellular DNA against agents-induced oxidative damage [49]. Hence, it can be postulated that *J. curcas*, by virtue of its free radical scavenging capacity and DNA repairing capacity restore DNA damage induced by STZ. The DNA damage induced by STZ is reduced by *J. curcas*, extracts as measured by the decrease DNA fragmentation and gene expression alterations. The antioxidant activity of the *J. curcas* extracts is attributed to the hydrogen donating ability of phenolic compounds present in the extracts [50]. The present study declared that, treatment of diabetic rats with several extracts of *Jatropha curcas* especially PE and EA extracts decreased the alteration in the expression of diabetic related genes (IGF1R, IGFBP2, IGFBP3, GRIN2C and VEGFA) induced by STZ in diabetic-rats. Up to date, there are no data concerning the effect of *Jatropha curcas* on diabetic related genes. Diabetic rats revealed decrease in the expression of IGF1R, IGFBP2, IGFBP3 and VEGFA genes as well as increase in the expression of GRIN2C gene which agreed with our results [51]. Medicinal plant extracts contain valuable anti-diabetic agents and may involve one or more active components responsible for blood glucose reduction [52]. Thus, the hypoglycemic effect of *J. curcas* in this study may be linked to the presence of flavonoids and terpenes in the extracts regulate the expression of diabetic related genes. These compounds have been implicated in the antidiabetic activities of many plants [53]. In this study, the hypoglycemic action of *J. curcas* different extracts may be by potentiating the insulin effect, either by increasing the pancreatic secretion of insulin from the cells of langerhans islets or its release from bound insulin in which these biological actions are regulated by the expression of several genes responsible on the diabetes pathways [54].

This article can be downloaded from www.ijpbs.net

CONCLUSION

The application of *J. curcas* extracts reduced the high levels of adhesion molecules. The administration of *J. curcas* extracts exhibited relatively rats of DNA damage similar to these of control group. Beside ,*J. curcas* extracts altered the expression of diabetes related genes and appeared similar to these of the control group. It could be concluded that *J. curcas* extracts might be a promising regulator supplements for hyperglycemia and the expression of genes related DM.

CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Ahmed MB, Ahmed MI, Ismail HAS and Esmat I, Role of oxidized LDL (ox-LDL) and adhesion molecules (VCAM-1, ICAM-1) in type 2 diabetes mellitus with a potential for atherosclerotic complications in patients of Qassim region. Journal of Investigational Biochemistry, 1(1):48-54, (2012).
2. Madan B, Prasad AK, Parmar VS and Ghosh B, 1,4-dihydroxyxanthone modulates the adhesive property of endothelial cells by inhibiting intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. Bioorganic & Medicinal Chemistry, 15;12(6):1431-7, (2004).
3. Moreli JB, Santos JH, Rocha CR, Damasceno DC, Morceli G, Rudge, MV, Bevilacqua E and Calderon1 IMP, DNA damage and its cellular response in mother and fetus exposed to hyperglycemic environment. Hindawi Publishing Corporation, 1-9, (2014).
4. Agnez-Lima LF, Melo JTA, Silva AE, Oliveira AHS, Timoteo ARS, Lima-Bessa KM, Martinez GR, Medeiros MHG, Mascio PD, Galhardo RS and Menck CFM, DNA damage by singlet oxygen and cellular protective mechanisms. Mutation Research, 751(1):15-28, (2012).
5. Iyama T and Wilson DM, DNA repair mechanisms in dividing and non-dividing cells. DNA Repair, 12(8):620-636, (2013).
6. Amran AA, Zakaria Z, Othman F, Das S, Al-Mekhlafi HM and Nordin NA, Changes in the vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and C-reactive protein following administration of aqueous extract of piper sarmentosum on experimental rabbits fed with cholesterol diet. Lipids in Health and Disease, 1-8, (2011).
7. Nishikawa T, Sasahara T, Kiritoshi S, Sonodak, Senokuchi T, Matsuo T, Kukidome D, Wake N. Matsumura T, Miyamura N. Sakakida M, Kishikawa H and Araki E, Evaluation of urinary 8-hydroxydeoxy-guanosine as a novel biomarker of macrovascular complications in type 2 diabetes. Diabetes Care, 26, 1507-1512, (2003).
8. Mushtag M and Wani SM, Polyphenols and human health – a review. International Journal of Pharma and Bio Sciences, 4(2):338-360, (2013).
9. Audipudi AV and Bhaskar VC, Antioxidative and antimicrobial activity of methanol and chloroform extracts of *Gmelina arborea* roxb. International Journal of Biotechnology and Biochemistry, 6, 139-144, (2010).
10. Kinsella J, Frankel E, German B and Kanner J, Possible mechanisms for the protective role of antioxidants in wine and plant foods. Food Technology, 47, 85-89, (1993).
11. Abd-Alla HI, Albalawy MA, Aly HF, Shalaby NM, Shaker AH, Flavone composition and antihypercholesterolemic and antihyperglycemic activities of *Chrysanthemum coronarium* L. Zeitschrift für Naturforschung, 69, 199-208, (2014).
12. Edoga HO, Okwu DE and Mbaebie BO, Phytochemicals constituentsof some Nigerian medicinal plants. African Journal of Biotechnology, 4(7):685-688, (2005).

13. Agbogidi OM and Eruotor PG, Morphological changes due to spent engine oil contamination and its heavy metal components of *Jatropha curcas.* In: Baby, S. and Sandhu, P.S. eds. Proceedings of the International Conference on Bioscience, Biotechnology and Health Sciences ICBBHs' 2012organized by Planetary Science Centre Research December 14 and 15, 2012 in Singapore, 88-93, (2012).
14. Mishra SB, Vijayakumar M, Ojha SK and Verma A, Antidiabetic effect of *Jatropha curcas* L. leaves extract in normal and alloxan-induced diabetic rats. International Journal of Pharmaceutical Sciences, 2(1):482-87, (2010).
15. Mohamed NZ, Abd-Alla HI, Aly HF, Mona M, Nehal I and Hassan SA, CCl_4-induced hepatonephrotoxicity: protective effect of nutraceuticals on inflammatory factors and antioxidative status in rat, Journal of Applied Pharmaceutical Science, 4, 087-100, (2014).
16. Shalaby NM, Abd-Alla HI, Aly HF, Albalawy MA, Shaker KH and Bouajila J, Preliminary *in vitro* and *in vivo* evaluation of antidiabetic activity of *Ducrosia anethifolia* Boiss. and its linear furanocoumarins, BioMed Research International, 2014, 1-13. (2014).
17. Dachicourt N, Bailb D, Gangnerou MN, Serradas P, Ravel D and Portha B, Effect of gliclazide treatment on insulin secretion and beta-cell mass in non insulin dependent, Goto kakisaki rats, European Journal of Pharmacology, 361, 243-251, (1998).
18. Burton K, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochemical Journal, 62, 315-323, (1956).
19. Lu T, Xu Y, Mericle MT, Mellgren RL, Participation of the conventional calpains in apoptosis. Biochimica et Biophysica Acta, 1590, 16-26, (2002).
20. Khalil WKB and Booles HF, Protective role of selenium against over-expression of cancer-related apoptotic genes induced by o-cresol in rats. Archives of Industrial Hygiene and Toxicology, 62, 121-129, (2011).
21. Gibbons GH and Dzau VJ, The emerging concept of vascular remodeling. New England Journal of Medicine, 330, 1431-1439, (1994).
22. Inoue I and Katayama S, The possible therapeutic actions of peroxisome proliferator-activated receptor alpha (PPAR alpha) agonists, PPAR gamma agonists, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, angiotensin converting enzyme (ACE) inhibitors and calcium (Ca)-antagonists on vascular endothelial cells. Current Drug Targets - Cardiovascular & Hematological Disorders, 4, 35-52, (2004).
23. Mojcik CF and Shevach EM, Adhesion molecules: a rheumatologic perspective. Arthritis & Rheumatology, 40, 991-1004, (1997).
24. Buck CA, Immunoglobulin superfamily: structure, function and relationship to other receptor molecules. Seminars in Cell Biology, 3, 179-88, (1992).
25. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME and Lobb RR, VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell, 60, 577-84, (1990).
26. Cui W and Song Y, Potential clinical usefulness of endothelial adhesion molecules in predicting risk of type 2 diabetes. North American Journal of Medicine and Science, 2(2):60-63, (2009).
27. Targher G, Bertolini L, Zenari L, Cacciatori V, Muggeo M, Faccini G and Zoppini G, Cigarette smoking and plasma total homocysteine levels in young adults with type 1 diabetes. Diabetes Care, 23, 524-528, (2000).
28. Moncada S and Higgs A, The L-arginine-nitric oxide pathway. New England Journal of Medicine, 30(329):2002-2012, (2002).
29. Shah BR and Hux JE, Quantifying the risk of infectious diseases for people with diabetes. Diabetes Care, 26, 510-513, (2003).

30. Libby P and Hansson GK, Involvement of the immune system in human atherogenesis: Current knowledge and unanswered questions. Laboratory Investigation, 64, 5-15, (1999).
31. Ceriello A, Falleti E, Motz E, Taboga C, Tonutti L, Ezsol Z, Gonano F and Bartoli E, Hyperglycemia-induced circulating ICAM-1 increase in diabetes mellitus: the possible role of oxidative stress. Hormone and Metabolic Research (Journal), 30(146):14-9, (1998).
32. Khalfaoui T, Lizard G and Ouertani-Meddeb A, Adhesion molecules (ICAM-1 and VCAM-1) and diabetic retinopathy in type 2 diabetes. Journal of Molecular Histology, 39(2):243-249, (2008).
33. Chen YH, Lin SJ, Ku HH, Shiao MS, Lin FY, Chen JW and Chen YL, Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF-alpha-treated human aortic endothelial cells. Journal of Cellular Biochemistry, 82, 512-521, (2001).
34. Ding M, Zhao GR, Yuan YJ and Guo ZX, Aqueous extract of *Salvia miltiorrhoza* regulates adhesion molecule expression of tumor necrosis factor alpha-induced endothelial cells by blocking activation of nuclear factor kappaB. The Journal of Cardiovascular Pharmacology, 45, 516-524, (2005).
35. Zhou Z, Liu Y, Miao A and Wang S, Protocatechuic aldehyde suppresses TNF-a-induced ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells. European Journal of Pharmacology, 513, 1-8, (2005).
36. Narayani M, Johnson M, Sivaraman A and Janakiraman N, Phytochemical and antibacterial studies on *Jatropha curcas* L. Journal of Chemical and Pharmaceutical Research, 4(5):2639-2642, (2012).
37. Knekt P, Jarvinen R, Seppanan R, Heliovaara M, Teppo L, Pukkala E and Aromaa A, Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. American Journal of Epidemiology, 146, 223-230, (1997).
38. Kay CD, Kris-Etherton PM and West SG, Effects of antioxidant-rich foods on vascular reactivity: review of the clinical evidence. Current Atherosclerosis Reports, 8, 510-522, (2006).
39. Matough FA, Budin SB, Hamid ZA, Alwahaibi N and Mohamed J, The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos University Medical Journal, 12(1):5-18, (2012).
40. Zhu QY, Hackman RM, Ensunsa JL, Holt RR and Keen CL, Antioxidative activities of Olong tea. Journal of Agricultural and Food Chemistry, 50, 6929-6934, (2002).
41. Sundari J, Selvaraj R, Prasad NR and Elumalai R, *Jatropha curcas* leaf and bark fractions protect against ultraviolet radiation-B induced DNA damage in human peripheral blood lymphocytes. Environmental Toxicology and Pharmacology, 36(3):875-82, (2013).
42. Linjawi SAA, Khalil WKB and Salem LM, Detoxified *Jatropha curcas* kernel meal impact against benzene-induced genetic toxicity in male rats. International Journal of Pharmacy, 4(4):57-66, (2014).
43. Boussageon R, Bejan-Angoulvant T, Saadatian-Elahi M, Lafont S, Bergeonneau C, Kassaï B, Erpeldinge S, Wright JM, Gueyffier F and Cornu C, Effect of intensive glucose-lowering treatment on all cause mortality, cardiovascular death, and microvascular events in type 2 diabetes mellitus: a meta-analysis of randomized controlled trials. British Medical Journal, 343, 1-12, (2011).
44. Osawa T, Protective role of dietary polyphenols in oxidative stress. Mechanism of Ageing and Development, 111(2-3):133-139, (1999).
45. Sundari J, Selvaraj R and Rajendra PR, Antimicrobial and antioxidant potential of root bark extracts from *Jatropha curcas* (Linn). Journal of Pharmacy Research, 4(10):3743-3746, (2011).
46. Ligangli YH, Scott M, Jonathan M, John W and Minq Q, Free radical scavenging properties of wheat extracts. Journal of Agricultural and Food Chemistry, 50, 1619-1624, (2002).

47. Rajkumar V, Guha G and AshokKumar R, Evaluation of antioxidant activities of *Bergeniaciliata rhizome*. Records of Natural Products, 4(1):38-48, (2010).
48. Svobodova A, Zdarilova V and Vostalova J, *Lonicera caerulea* and *Vaccinium myrtillus* fruit polyphenols protect HaCaT keratinocytes against UVB-induced phototoxic stress and DNA damage. Journal of Dermatological Science, 56(3):196-204, (2009).
49. Vostalova J, Zdarilova A and Svobodova A, *Prunella vulgaris* extract and rosmarinic acid prevent UVB-induced DNA damage and oxidative street in HaCaT keratinocytes. Archives for Dermatological Research, 302(3):171-181, (2010).
50. Dhawan A, Anderson D, Pascaul-Teresa S, Santos-Buelga C, Clifford MN and Ioannides C, Evaluation of the antigenotoxic potential of monomeric and dimeric flavanols, and black tea polyphenols against heterocyclic amine-induced DNA damage in human lymphocytes using the Comet assay. Mutation Research, 515, 39-56, (2002).
51. Lau JCM, Kroes RA, Moskal JR and Linsenmeier RA, Diabetes changes expression of genes rel ated to glutamate neurotransmission and transport in the long-evans rat retina. Molecular Vision, 19(19):1538-1553, (2013).
52. Grover JK, Yadav S and Vats V, Medicinal plants of India with hypoglycemic potentials. Journal of Ethnopharmacology, 81, 81-100, (2002).
53. Okokon JE, Ita BN and Udokpoh AE, The *in vivo* anti-malarial activities of *Uvariae chamae* and *Hippocratea africana*. Annals of Tropical Medicine and Parasitology, 100(9):585-590, (2006).
54. Pari L and Amarnath S, Antidiabetic activity of *Boerhavia diffusa* L. effect on hepatic key enzymes in experimental diabetic rats. Journal of Ethnopharmacology, 91, 109-113, (2004).