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Effect of a Mixture of Plants Extracts on Genomic DNA, Insulin Receptor, and Insulin Receptor Substrate-1 Genes in Alloxan-Induced Diabetic Male Rats.

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Abstract
This study was designed to study the effects of methanol-watery extracts mixture of five selective medicinal plants on genomic DNA, insulin receptor, and insulin receptor substrate-1 genes in alloxan-induced diabetic male rats. The molecular assays used in this study was DNA fragmentation test and polymerase chain reaction of insulin receptor and insulin receptor substrate-1. Phytochemical assays (thin layer chromatography (TLC) and antioxidant activity by β-carotene spray) were carried out for characterization of methanol-watery extracts of each plants used (*Trigonella faenum-graecum* (fenugreek) seeds, *Nigella sativa* (black cumin) seeds, *Zingiber officinale* (ginger) rhizomes, *Olea europeae* (olive) leaves, and *Fraxinus ssp.*(ash) seeds). The results indicate presence of number of antioxidant bands with different retardation factors (R_f). Molecular study of DNA showed fragmentation in both DNA extracted from WBC and renal cortex in diabetic group while treatment with mixture of plants extracts significantly reduced DNA fragmentation in both DNA extracted from WBC and renal cortex in all treatment groups except treatment for 45 days which still had lyses level 5000 bp of DNA extracted from renal cortex. On the other hand, insulin receptor gene of diabetic and diabetic-treated groups for different periods (45, 60, 75 days) showed precense of one band (200 bp) compared with normal control which had two bands while insulin receptor substrate-1 has normal band (320 bp) in normal and treated groups.
Keywords: alloxan, insulin receptor, insulin receptor substrate -1, antioxidant, DNA fragmentation

1. Introduction

Recently, the search for appropriate hypoglycemic agents has been focused on plants used in traditional medicine (Rates, 2001). Medicinal plants are frequently considered to be less toxic and free from side effects than the synthetic ones (Santhakumari *et al*., 2006).

The insulin receptor (IR) is a transmembrane receptor that is activated by insulin (Sciacca, 2003). The human insulin-receptor (hlNSR) gene spans a region of >120,000 base pairs (bp) on the short arm of chromosome 19. It is comprised of 22 exons or coding regions that vary in size from 36 to >2500 bp. To a large degree, the introns appear to divide the hlNSR gene into segments that encode structural and/or functional elements of the hlNSR protein. Many mutations in the hlNSR gene that result in expression of structurally abnormal proteins have been described. These mutations are associated with insulin resistance and provide insight into the role of the hlNSR gene in the development of DM (Seino *et al.,* 1990).

The IR composed of two α-subunits and two β-subunits. The α-subunit is primarily extracellular and contains the ligand-binding domain, whereas the β-subunit is intrinsic to the lipid bilayer and contains the tyrosine kinase signaling domain, which catalyzes the transfer of the phosphate of ATP to tyrosine residues on protein substrates (De Meyts, 2004). The IR occurs as two alternatively spliced isoforms, IR-A (exon 11−) and IR-B (exon 11+)(Norgren *et al*.,1994; Serrano *et al.,* 2005; Nevado *et al.,* 2008), which exhibit functional differences and are expressed in a tissue-specific manner(Serrano *et al.,* 2005).

Patients with diabetes mellitus type II suffer from hyperglycemia because they are not able to use the insulin that they produce, often due to inadequate function of insulin receptors. There are some evidences that this deficiency is inherited and leads to the malfunction of the pancreatic beta cells resulting in insulin excretion disorders (Kazemi *et al.,* 2009). The IR was considered one of candidate gene for Type 2 diabetes (Sesti, 2001). Mutations in the insulin receptor gene can render the cell resistant to the biological action of insulin .One of these mutation encoding substitution of valine for phenylalanine at position 382 in the α-subunit of the insulin (Accili *et al*., 1989). In addition, the investigations of a patient with a genetic form of insulin resistance, have identified a mutant allele encoding an

insulin receptor in which lysine is substituted for asparagine at position 15 of the α-subunit (Kadowaki *et al.*, 1990).

The insulin receptor substrate (IRS) proteins are critical to signal transduction in insulin target tissues (Sesti *et al.,* 2001). The IRS-1, IRS-2, IRS-3, and IRS-4 have been shown to play unique roles in insulin signal transduction (Laustsen *et al.,* 2002) and differ in function and tissue distribution (Serrano *et al.,* 2005). The substrate proteins that are phosphorylated by the IR include a protein called "IRS-1". IRS-1 binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (Glut4) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissue, and therefore to an increase in the uptake of glucose from blood into these tissues (Sciacca *et al*., 2003).

Furtheremore, IRS serve as messengers from activated cell surface receptors to numerous signaling pathway cascades. One of these pathways, phosphoinositide 3-kinase (PI3K), and serve a guide to using targeted pathway therapy (Metz and Houghton, 2010). Activation of PI3K by phosphorylated IRS-1 also leads to activation of glycogen synthase through a process that involves activation of protein kinase B/Akt resulting in the stimulation of glucose transport. The action of insulin to increase protein synthesis and inhibit protein degradation also is mediated by PI3K (Saltiel and Kahn, 2001).

A genetic predisposition for T2DM, exhibit a reduced IRS-1 protein expression in the adipocytes. These individuals are markedly insulin resistant, which suggests an increased risk for type 2 diabetes. IRS-1 and GLUT4 gene expression levels were also lower in diabetic, and this may also be a marker for individuals who eventually develop type 2 diabetes (Carvalho *et al.,* 2001). Previous study reported that diabetic patients carrying the Arg972 insulin receptor substrate-1 (IRS-1) variant are at increased risk for secondary failure to sulfonylurea (Sesti *et al.,* 2004). Also, the common Gly^{972}-Arg amino acid polymorphism of insulin receptor-1(Arg^{972} IRS-1) has been associated with human T2DM (Marchetti *et al.,* 2002).

2. MATERIALS AND METHODS

2.1. Phytochemical assays :

2.1.1. Plant extract preparation: The plants which used in this study were purchased from a local herbal markets except olive leaves were collected from gardens of Babylon university. The plants parts used in this study were *Trigonella faenum-graecum* (fenugreek) seeds, *Nigella sativa* (black cumin) seeds, *Zingiber officinale* (ginger) rhizomes, *Olea europeae* (olive) leaves, and *Fraxinus ssp.*(ash) seeds. Oilve leaves were rised with water to remove dust, insecticides, and contaminated materials then dried in dark. All plants materials were ground into fine powder.

The plants extracts were prepared according to Sato *et al*. (1990). Each plant powder homogenize with solvent mixture (methanol: distal water) (20:80 v\v) in blander for 30 min , the mixture are infiltration and dry in oven 45 ^{o}C for 24 hours , the product store in dark container. The mixture was prepared immediately by mixing 0.5 ml of each extract (concentrations graded 10-100 mg /kg body weight).

2.1.2. Plant extract characterization by TLC: by use many solvents:

A- Methanol: ethyl acetate: DW (20:60:20 V\V\V) as mobile phase for fenugreek seeds, black cumin seeds, and olive leaves.

B- Hexan: diethyl ether (40:60 V/V) for ginger.

C- Chloroform: methanol: distilled water (52:40:8 V/V/V) for fraxinus.

2.1.3. Antioxidant activity : this test performed by use β-caroten spray (prepare by dissolve 9 gm of β-carotene in 30 ml chloroform and 2 drop of linolic acid with 60 ml ethanol) (Pratt and Miller, 1984). TLC was spry by this mixture and left it in light for 6 hour; the bands have yellow colure for longer time was antioxidant activity.

2.2. Induction of diabetes: Injection of alloxan multiple doses of 120 mg/kg i.p was used for the induction of diabetes mellitus type 2 (T2DM). 36 albino male rats were used and randomly divided into six groups (n=6 each group) included; group I: normal negative control, group II: diabetic control, group III: normal rats treated with plant extract mixture for 60 days, group IV: diabetic rats treated with plant extract mixture for 45 days, group V: diabetic rats treated with plant extract mixture for 60 days, and group VI: diabetic rats treated with plant extract mixture for 75 days.

2.3. DNA extraction: according to promega (USA) kit leaflet. The concentration and purity of DNA was calculated by using nanodrop (Cleaver scientific -Japan).

2.4. DNA fragmentation test (DFT): by DNA electrophoresis in agarose gel, then the gel was exposed to UV light 260 nm and pictures was taken (Robinson and Lafleche ,2000).If The fragment was smear therefore lyses level was calculated according to distance between beginning and end the smear compare with negative control.

2.5. Polymerase chain reaction (PCR)

A total of 25 µl reaction volume in each tube contained genomic DNA, 40 pmol each of forward and reverse primers, and 12.5 µl Green Master Mix (Promega). Termal cycle conditions was as in table -2.

The relative size of the PCR products were determined using 1250 bp DNA ladder (Cleaver scientific-Japan)**.** The PCR products were electrophoresed in 1.5% agarose gel. The gel was stained with ethidium bromide, and the bands were visualized under UV transillumination and photographed .

3.Results

3.1:Plant extract characterization by TLC

TLC profile and β-carotene spray results showed presence of number of antioxidant bands with different retardation factors (R_f) as in figure (1) and table (3)**.**

3.2:DNA Fragmentation Test (DFT)

3.2.1: DNA extraction from white blood cells

Figure (2) and table (4) showed electrophoresis of DNA extracted from white blood cells of experimental groups .In diabetic group, DNA electrophoresis gave a smear which has lyses level about 9500 bp and had molecular size about 10000-500 bp . All diabetic groups treated with mixture of plants extracts for 45,60,75 days and normal groups treated with mixture of plants extracts for 60 days had normal DNA and their molecular size was 10000bp which was the same of the DNA of negative control group.

3.2.2: DNA extracted from renal cortex

Figure (3) and table (5) showed electrophoresis of DNA extracted from renal cortex of experimental groups. Alloxan –induced diabetes in experimental rats caused lyses of DNA which appear as a smear have molecular size 10000-500 bp and lyses level was 9500 bp compared with negative control which have normal DNA band. Mixture of plants extracts did not cause lyses of DNA in normal rats while the diabetic group treated for 45 days had lyses level of DNA which appear as smear with lyses level 5000 bp and have molecular size (10000-5000 bp). Diabetic group treated for 60 and 75 days had normal DNA bands and their molecular size was as DNA of negative control group.

3.4:Polymerase chain reaction (PCR) analyses

3.4.1:The insulin receptor (IR) gene:

The IR gene was ampilified and all amplicons were resolved on a 1.5 % agarose for 1 hour at 70 volt in 1X TBE and stained ethidium bromide to confirm successful ampilification and presented in figure 4-18.This photograph were taken after exposure to UV light. Fragment sizes derived from the 1250 bp DNA marker loaded in the first lane. As seen in figure (4),the amplicon of IR gene of negative control group have two bands which are 490 bp and 200 bp .Moreover, in diabetic group and diabetic group treated with plant extract mixture for 45,60,75 days had only one band which had MW 200 bp.

3.4.2:The insulin receptor substrate-1 (IRS-1) gene

The IRS-1 gene was ampilified and all amplicons were resolved on a 1.5 % agarose for 1 hour at 70 volt in 1X TBE and stained ethidium bromide to confirm successful ampilification and presented in figure 4-19.This photograph were taken after exposure to UV light.Fragment sizes derived from the 1250 bp DNA marker loaded in the first lane .The amplicon of IRS-1 gene of negative control group have one band which have molecular size 320 bp in all experimental groups.

4.DISCUSSION

4.1:DFT of both DNA extracted from WBC and renal cortex

In the group injected with alloxan, DNA had a lyses level about 9000 bp and 9500 bp of DNA extracted from WBC and renal cortex respectively. This result may due to alloxan induce ROS formation and oxidative stress and hence DNA strand fragmentation, breaks and loss of chromosome integrity as mentioned by (Cinkilica *et al*., 2009).

The daily intake of mixture for normal and diabetic groups treated for different period gives normal DNA bands

of both DNA extracted from WBC and renal cortex except DNA extracted from renal cortex of diabetic group treated for 45 days have lyses level of about 5000 bp .These results due to the ability of plant extract mixture to act synergistically to maintain genomic stability or its ability to induction DNA repair systems due to antioxidant activities which reduce or prevent generation of free radicals. Also, the longer period of treatment (60 and 75) days is more effective in genomic stability and induction of DNA repair.

4.2:PCR assay

4.2.1:IR gene

The detection of two discrete bands of PCR amplicon of IR gene from renal cortex tissue sampled of negative control group which has molecular size 490 bp and 200 bp respectively. In human IR (hINSR) gene is comprised of 22 exons or coding regions that vary in size from 36 to >2500 bp. To a large degree, the introns appear to divide the hlNSR gene into segments that encode structural and/or functional elements of the hlNSR protein (Seino *et al*., 1990). The IR occurs as two alternatively spliced isoforms, IR-A (exon 11−) and IR-B (exon 11+), which exhibit functional differences and are expressed in a tissue-specific manner (Norgren *et al*., 1994; Serrano *et al*., 2005).

Moreover, in diabetic group and diabetic group treated with plant extract mixture for 45,60,75 days, PCR amplicon of IR gene had only one band which had molecular size 200 bp. The absence of 490 bp band in alloxan –induced diabetic group may due to reactive oxygen species produced from alloxan cause DNA strand breaks as mentioned by (Okamoto, 1985) and this absence of one band may cause defects in insulin binding to its receptors or post-receptor defect including insulin signal transduction abnormalities and decreased glucose transport in kidney cells reflecting in degeneration in kidney histological structure and physiological function as urea levels significantly increased in diabetic group.

Oxidative stress due to chronic hyperglycemia leads to the generation of reactive oxygen species (ROS) and loss of chromosomal integrity (Boehm *et al*., 2008). Free radicals especially increased generation of nitric oxide (NO) in diabetic state may also affect the formation of the insulin receptor complex. Another possibility is that the receptor undergoes post-translational modification that alters binding and signal tansmission properties (Kasuga *et al*., 1982). It is therefore, a possibility that over production of free radicals due to lipid peroxidation may alter the interaction of insulin with its receptors thus affecting the ability of insulin to differentially regulate its receptor and the regulator proteins (Harmon *et al*.,1980). The lower number of receptor binding sites per cell in diabetic rats could be the result of primary alteration in the receptor or might be secondary to alteration in integrity of the membrane (Slater 1984). The resulting membrane dysfunction can impair transport of glucose across the membrane which leads to the observed hyperglycemia (Pari *et al*., 2007).

Also, western blot analyses demonstrated a significant reduction in the expression of insulin receptor subunits in the kidney compared to lean control rats and insulin receptors are downregulated in the kidneys of insulin resistant rats, possibly mediated by hyperglycemia and angiotensin II (Tiwari *et al*., 2007). Our result go in coincidence with previous study which revealed that the group subjected to alloxan showed decrease in DNA content in the kidney compared to the control group, the alloxanated diabetic rats showed decrease in protein content in renal tubules of kidney. This decrease may be due to a decrease in ribosomal granules of rough endoplasmic reticulum or due to a decrease in DNA content. The decrease of DNA content was associated with a decrease in protein content in kidney cells of diabetic rats (Shaffie *et al*.,2010)

The absence of larger band of IR gene in diabetic groups treated for different periods of study may be because the Mixture of Plants Extracts need more time than 75 days to cause repairing of DNA consisting IR gene or may cause changing in the functional specifity of insulin /IR signaling and post –receptor events. The mixture may cause effects on another receptors as insulin-like growth factor receptor (IGFR) or insulin receptor-related receptor (IR-R), a novel member of the insulin receptor family IR-R, this IR-R have a characteristic localization of IR-R mRNA at the cellular level in the kidney, stomach and brain and the involvement of IR-R in the physiological functions of insulin-secreting cells. IR-R transcripts are present at much higher levels in the kidney than in any other tissues (Ozaki,1998) or may has effect on other candidate genes for DM.

4.4.2: IRS-1 gene

IRS-1 gene does not affected by alloxan and other treatment, this may be due to effect of alloxan on others IRSs genes such as IRS-2, IRS-3, IRS-4 which play a role in insulin receptor signaling (Jhala *et al*., 2003).

Insulin receptor substrate-2 (IRS-2) belongs to a family of cytoplasmic adaptor proteins, which link insulin,

IGF-1, and cytokine receptor tyrosine kinases to signaling pathways regulating metabolism, growth, and differentiation (White,1997). IRS-2–deficient mice display all characteristics of type 2 diabetes, suggesting that dysfunction of the IRS-2 gene may contribute to the pathogenesis of human type 2 diabetes (Wither *et al*.,1998).

Finally, alloxan may be affect on other genes, since T2DM is a complex heterogenous disease with multiple genes contributing to the cause of this disorder. It may affect on other genes controlling carbohydrates and lipid metabolism.

5. Conclusion

In conclusion, using mixture of plants extracts used in this study could prevent or reduce molecular changes caused by type II DM.

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Table (1): Sequences of PCR primers (Serrano *et al*., 2005).

Gene	PCR primers	Length
IR	5'-TTCATTCAGGAAGACCTTCGA-3' 5'-AGGCCAGAGATGACAAGTGAC-3'	21 mer 21mer
IRS-1	5'-GCCAATCTTCATCCAGTTGC-3' 5'-CATCGTGAAGAAGGCATAGG-3'	20 mer 20 mer

Table (2): Thermal cycle conditions.

Stages	Cycle	Step	Temperature	Time	Function
1	1	1	94	5 min	Initial denaturation
2	30	1	94	2 min	DNA denaturation
		2	45	0.45 sec	Primer annealing
		3	72	1 min	Template elongation
3	1	1	72	7 min	Final elongation
		2	4	hold	Incubation

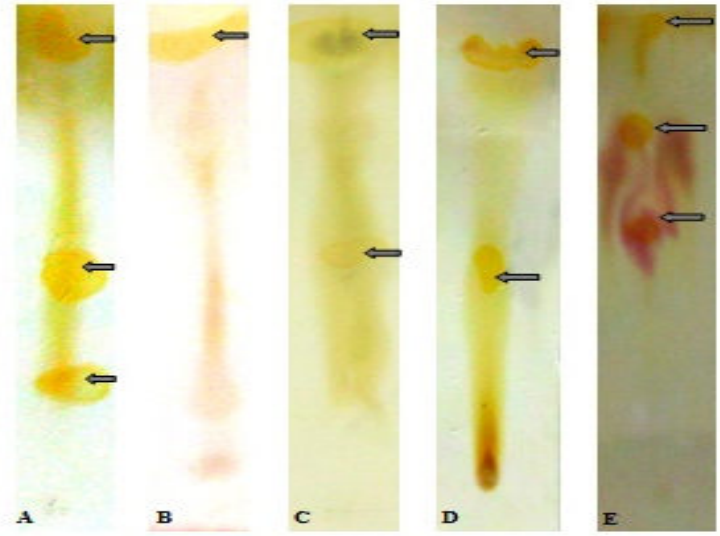


Figure (1): Antioxidant bands of methanol – watery extract of plants used.
A. Fenugreek seeds. B. Black cumin seeds. C. Olive leaves. D. Ginger rhizome.
E. Fraxinus seeds.

Table (3): TLC of methanol – watery extract of fenugreek seeds using β-carotene spray.

Methanol-watery extract of plant	R_f	Number of bands
fenugreek seeds	0.205	3
	0.370	
	0.823	
Black cumin seeds	0.629	1
Olive leaves	0.445	2
	0.903	
Ginger rhizome	0.483	2
	0.928	
Fraxinus seeds	0.516	3
	0.735	
	0.787	

Table (4): Lyses level of DNA extracted from white blood cells compared with negative control.

Lane no.	Treatment	Base pair of band	Lyses level compared with negative control
1	DNA marker	10000	_
2	Negative control	10000-10000	_
3	Diabetic control	10000-1000	9000
4	Mixture of plants extracts (60 days)	10000-10000	_
5	DM + Mixture of plants extracts (45 days)	10000-10000	_
6	DM + Mixture of plants extracts (60 days)	10000-10000	_
7	DM + Mixture of plants extracts (75 days)	10000-10000	_

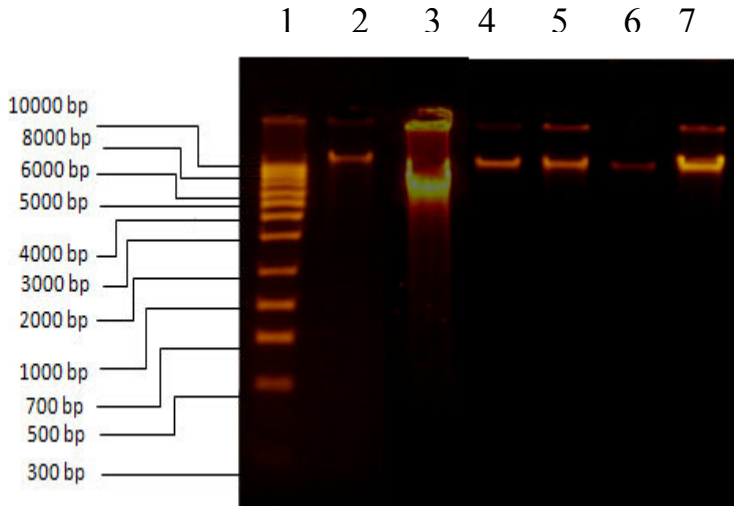


Figure (2): Electrophoresis of DNA extracted from white blood cells of experimental groups. Lane: DNA ladder (1 kb), lane 2: Negative control, lane 3: Diabetic control, lane 4: Mixture of plants extracts (60 days), lane 5: DM +Mixture of plants extracts (45 days), lane 6:DM +Mixture of plants extracts (60 days), lane 7: DM +Mixture of plants extracts (75 days).

Table (5): Lyses level of DNA extracted from renal cortex compared with negative control.

Lane no.	Treatment	Base pair of Band	Lyses level compared with negative control
1	DNA marker	10000	_
2	Negative control	10000-10000	_
3	Diabetic control	10000-500	9500
4	Plant extracts mixture(60 days)	10000-10000	_
5	DM + Mixture of plants extracts(45 days)	10000-5000	5000
6	DM + Mixture of plants extracts(60 days)	10000-10000	_
7	DM + Mixture of plants extracts(75 days)	10000-10000	_

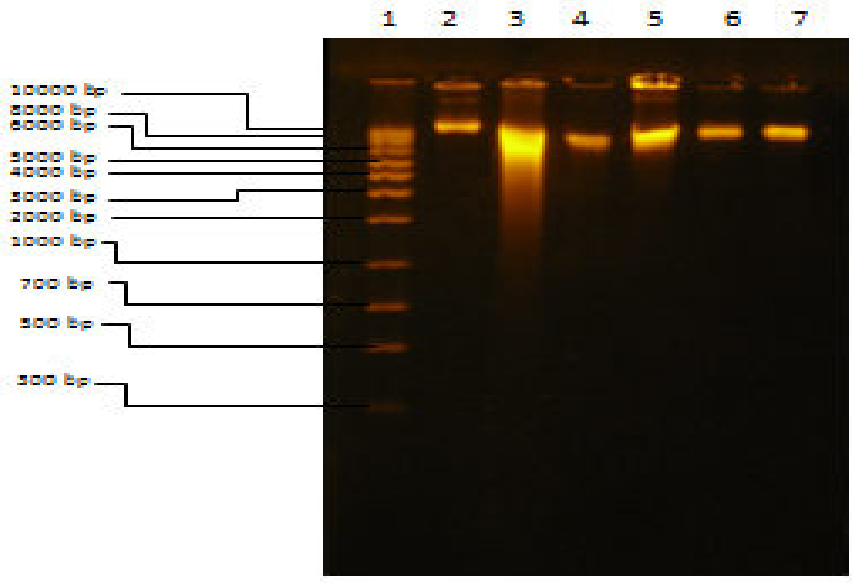


Figure (3): Electrophoresis of DNA extracted from renal cortex of experimental groups. Lane1: DNA ladder (1 kb), lane 2: Negative control, lane 3: Diabetic control, lane 4: Mixture of plants extracts (60 days), lane 5: DM +Mixture of plants extracts (45 days), lane 6:DM +Mixture of plants extracts (60 days), lane 7: DM +Mixture of plants extracts (75 days).

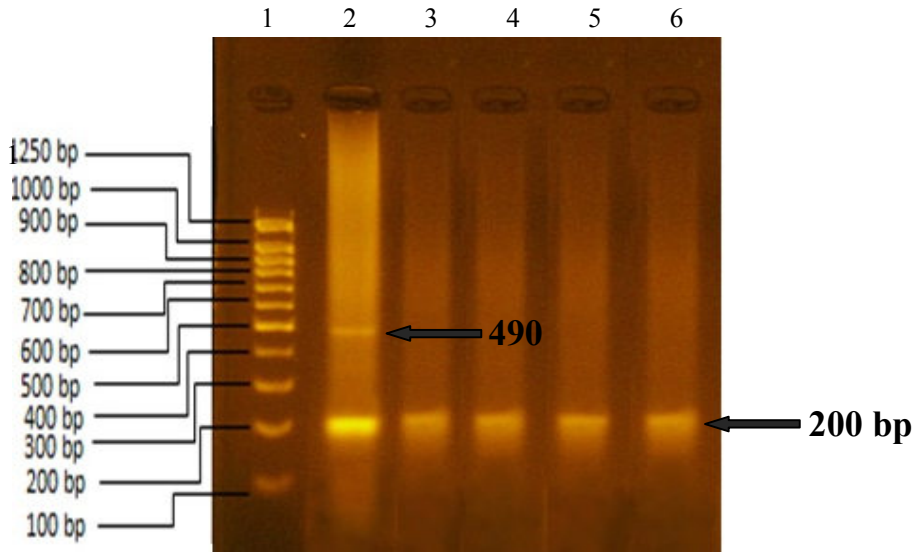
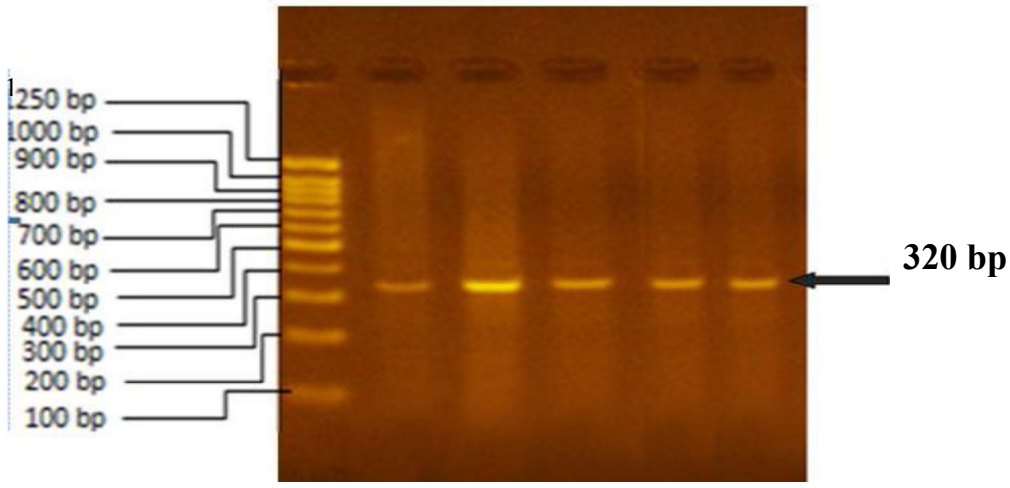


Figure (4): Electrophoresis of PCR product after IR gene amplification .Two bands are 490 bp and 200 bp detected in negative control and only one band 200bp in diabetic, normal and diabetic – treated groups with mixture for different periods of study. Lane 1: DNA ladder (1250 bp), lane 2: negative control, lane 3: diabetic control, lane 4: diabetic control treated with the mixture for 45 days, lane 5: diabetic control treated with the mixture for 60 days, lane 6: diabetic control treated with the mixture for 75 days.



Figure(5):Electrophoresis of PCR product after IRS-1 gene amplification. One band 320 bp is detected in negative control, diabetic, normal and diabetic – treated groups with mixture for different periods of study. Lane 1: DNA ladder (1250 bp), lane 2: negative control, lane 3: diabetic control, lane 4: diabetic control treated with the mixture for 45 days, lane 5: diabetic control treated with the mixture for 60 days, lane 6: diabetic control treated with the mixture for 75 day.

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