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Moringa oleifera in Vitro Culture and its Application as Anti-Diabetic in Alloxan Induced Diabetic Albino Mice

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A B S T R A C T

This work is aimed to indicate the anti-diabetic effect of ethanol extract of *Moringa oleifera* leaves and callus tissues in alloxan induced diabetic albino mice. Eighteen albino mice were randomly grouped into eight groups. The first and second one were positive and negative groups, other six groups were treated with ethanol extract in different concentration (20%, 40% and 80%) for both leaves and callus tissues. Results revealed that the highest mean callus growth obtained at 0.5mg/l BA and 2.5mg/l 2,4-D (779.5mg). Results also showed that ethanol extract of callus tissues significantly reduce glucose level in the blood of the treated albio mice recording 113.3 mg/Ld at the day 15 of the treatment. Weight of the treated animals significantly increased in all treatment begin from day 7 compared with negative control.

Introduction

Diabetes mellitus is a prevalent disease affecting the population of most of the countries in the world. This disease is caused by the abnormality in carbohydrate metabolism which could be due to low insulin secretion or insensitivity of target organs to insulin (Sugunabai *et al.*,, 2014). In diabetes, lipid abnormalities, anemia, alteration of liver and kidney functional indices has been implemented as major risk factors to the progression of both microvascular and macrovascular diabetes complications, people suffering from diabetes cannot produce or properly use insulin, and so they persistently have high blood glucose (Aja *et al.*,, 2015).

Plants are well known in traditional medicine for their hypoglycaemic effects and available literature indicates that there are more than 800 plant species showing hypoglycaemic activities *in vivo*. The maximum therapeutic and minimum side effects of herbal remedies have been verified in numerous scientific investigations. Also, plant materials play major roles in primary health care as therapeutic remedies in many

developing countries (Jogender *et al.*,, 2013).

It has become imperative to investigate plants such as *Moringa oleifera* and *Vernonia amygdalina* which have been used by native populations as hypoglycaemic agents in a standardized experimentation. A number of investigations have shown that saponins, flavonoids and a host of other secondary plant metabolites including arginine and glutamic acid possess hypoglycemic effect in various animal models and have been found to be hepatoprotective in diabetic animal experiments (Efiong *et al.*,, 2013).

Moringa oleifera has become very important so its contain 18 of the 20 amino acid that the human body required and is one of the plant species with higher seed oil content, between 30-40% (Cartes and Delaveau, 2014).

Tissue culture has been utilized extensively for mass propagation of economically important or elite plants, as well as species that are endangered due to extinction (Salema *et al.*,, 2007). *Moringa oleifera* callus induction was a greatly influenced by temperature, nutrients, pH and addition of ascorbic acid in the growing medium, Furthermore, the seeds contain an essential oil (Bennett *et al.*,, 2003). Stephenson and Fahey (2004) reported that 20% success rate of germination of immature seeds with subsequent shoot development from the epicotyl meristematic tissues of *M. oleifera* Lam. cultured on Murashige and Skoog (MS) semi-solid medium (Murashige and Skoog 1962) amended with 1 mg/L benzylaminopurine (BAP) and 1 mg/L gibberellic acid (GA3) and the success rate of seed germination was increased to 73% when immature seed explants were inoculated on membrane raft in MS liquid medium of the same media formulation, with the number of shoots regenerated roughly comparable to the semi-solid medium.

Materials and Methods

Materials

The chemicals and reagents used are of analytical quality. Fresh leaves of fully grown *Moringa oleifera* were collected from a green house at the college of applied biotechnology, Al-Nahrian university, Baghdad, Iraq.

Method

Callus Induction and Growth

Seeds of *M. oleifera* were washed with running water for 15 minutes; surface sterilized with 50% Clorox solution for 20 minutes, and then rinsed three times with sterile distilled water. The seeds after dipping in 99% ethanol, and removing the seed coats were cultured on MS agar medium containing 4.0 mg/l BA for seed germination, then shoot explants were cut into the small pieces and placed on the MS agar medium supplemented with 0.0, 0.5, 1.0 or 1.5mg/l BA and 0.0, 1.5, 2.0 or 2.5mg/l 2, 4-D for six weeks. All cultures were incubated at 25 ± 2ºC. Callus induction frequency (%) was calculated using the following formula (Yousif. 2002) .

(No. of seeds produced callus/total seeds cultured)*100. For callus growth, the induced calli (initial weight was 150 mg/tube) were sub-cultured, on the same medium.

Preparation of Extracts

The leaves and callus pieces of *Moringa oleifera* were dried in oven at 40ºC. The dried tissues were grinding well then the powder was soaked in 70% ethanol at room

temperature for 24 hours, filtered and evaporated to dryness using rotator evaporator.

Induction of Diabetes and Animals Treatment with Extracts

Alloxan 100 mg/kg of body weight was used to induce diabetes. Mice were injected intraperitoneally. The dose was prepared by dissolving 0.2ml alloxan in 0.9 NaCl solutions. A solution of 5% glucose was administered orally. Blood glucose was observed 20 hours after alloxanistion (Sushruta *et al.*,, 2006). In this study there was eight groups of treatment: +control group (non-treated with alloxan), -control group (treated with alloxan), other six groups treated with 20%, 40% and 80% either of *Moringa oliefera* leaves or callus ethanol extracts.

Blood Samples Collection and Determination of Animals Weight

The blood samples were collected from the tail vein puncture for measurement of blood glucose in all mice groups. Glucometer then used for determination the Glucose level**.** Animals weight were determined using sensitive balance.

Results and Discussion

Results displayed in table 1 revealed that 0.5 and 1.0 mg/l BA significantly increase the mean % callus induction recording 57.575 and 45.25% respectively compared with 0.0 mg/l BA (20.425%), the highest mean percentage of callus induction obtained at 0.5mg/l BA, due to the effect of 2,4-D the mean % callus induction increased significantly at 1.5, 2.0 and 2.5mg/l 2,4-D with mean values 38.3, 60.7 and 60% respectively compared with 0.0mg/l 2,4-D (0.0%) and the highest mean recorded at 2.0mg/l 2,4-D (60.7%). The interaction between 2,4-D and BA indicated that 0.5mg/l BA and 2.5mg/l 2,4-D recording the highest percentage of callus induction (93.5%). Data showed in table 2 exhibited that 0.5 and 1.0mg/l BA increased the mean callus fresh weight significantly (367.46 and 331.64mg respectively) compared with 0.0 and 1.5mg/l BA (168.82 and 288.16mg respectively) and the highest mean callus fresh weight obtained at 0.5mg/l BA while mean callus fresh weight increased significantly with the increasing 2,4-D concentration recording 0, 219.325, 341.95, 380.8 and 503.025 mg in 0.0, 1.0, 1.5, 2.0 and 2.5mg/l 2,4-D respectively. The interaction revealed that the highest mean callus fresh weight obtained at 0.5mg/l BA and 2.5mg/l 2,4-D with mean value 779.5mg, these results was agreed with those of Hussian *et al.,* (2010) who investigated that callus induction increased with increasing 2,4-D concentration. Callus, mainly comprising masses of undifferentiated cells, is good starting material for *in vitro* manipulation (Oriabi, 2013). But these results was disagreement with those obtained by Shank *et al.,* (2013) who reported that MS medium supplemented with 0.5mg/l of 2,4-D was the most effective medium for callus induction of *M. oleifera* with 100% of callus induction from week-3 after culturing of shoot, Figure 1 showing the callus mass of *M. oleifera* which grown on MS medium after two subcultures.

Results in table 3 described the changes in the weight of animals based on varying extract doses during the 15 days of treatment and showed that the body weight of mice significantly increased in the positive control group and those treated with 20, 40 and 80% of *M. oleifera* leaves or callus extracts compared with negative control which was significantly decreased with

increasing days of treatment, these results was agreement with those obtained by Aja *et al.,* (2013) who reported a significant (P<0.05) reductions in the mean body weight of rats in diabetic control compared to positive group while rats in treated groups showed significant (P<0.05) increase in their mean body weight compared to diabetic control group in work done earlier on anti-diabetic effect of aqueous extract of *Moringa oleifera* and *Bridelia ferruginea* leaves in alloxan-induced diabetic albino rats. Data displayed in table 4 show that administration of *Moringa oleifera* ethanol extracts of either leaves or callus tissues in alloxan induced diabetic albino mice at various doses of 20, 40 and 80% reduce the blood glucose level in the treated mice significantly compared to the negative control.

Table.1 Effect of the Interaction between 2,4-D and BA on the Mean of % Callus Induction, after Inoculating Explants onto Solid MS Medium for Four Weeks, n=30

2,4-D(g/l) / BA(mg/l)	0.0	1.5	2.0	2.5	Mean
0.0	**0**	**19.8**	**28.1**	**33.8**	**20.425**
0.5	**0**	**63.2**	**73.6**	**93.5**	**57.575**
1.0	**0**	**48.9**	**72.9**	**59.2**	**45.25**
1.5	**0**	**21.3**	**68.2**	**53.5**	**35.75**
Mean	**0**	**38.3**	**60.7**	**60**	
LSD 0.05	**2,4-D = 6.01; BA= 6.35; Interaction = 20.04**				

Table.2 Effect of 2,4-D and BA on the Mean Callus Fresh Weight (mg), after Inoculating Callus Pieces onto Solid MS Medium for Five Weeks. Initial Weight was 100mg. n= 30

2,4-D(g/l) / BA(mg/l)	0.0	1.0	1.5	2.0	2.5	Mean
0.0	**0**	**113.1**	**261.3**	**185.3**	**284.4**	**168.82**
0.5	**0**	**178.3**	**352.1**	**527.4**	**779.5**	**367.46**
1.0	**0**	**301.7**	**422.5**	**371.9**	**562.1**	**331.64**
1.5	**0**	**284.2**	**331.9**	**438.6**	**386.1**	**288.16**
Mean	**0**	**219.325**	**341.95**	**380.8**	**503.025**	
LSD 0.05	**2,4-D = 32.81; BA= 32.81; Interaction = 93.42**					

Table.3 Effect of Extract Doses on the Weight of Alloxan Induced Diabetic Albino Mice after 15 Days of Treatment, n=10

Type of Treatment / Days	mg/Ld + control	mg/Ld - control	Leaves extract			Callus extract			mg/Ld Mean
			20%	40%	80%	20%	40%	80%	
1	26.86	26.91	26.58	26.59	26.69	26.76	26.82	26.65	26.75
2	26.84	26.87	26.53	26.53	26.84	26.59	26.81	26.89	23.75
3	26.94	26.89	26.72	26.73	26.89	26.74	26.93	26.78	26.82
4	26.89	26.62	26.79	26.69	26.93	26.82	26.97	26.88	26.82
5	26.93	26.29	26.77	26.84	26.99	26.95	26.89	27.06	26.84
6	27.36	26.01	26.68	26.78	26.96	26.87	26.96	27.11	26.84
7	27.54	25.79	26.85	26.89	27.05	26.97	26.96	27.17	26.90
8	27.63	25.81	26.89	26.96	27.09	26.95	26.98	27.32	26.95
9	27.69	25.52	26.89	26.95	27.13	27.03	26.99	27.68	26.98
10	27.83	25.32	26.83	26.98	27.22	27.02	26.98	27.85	27.03
11	27.89	25.21	26.97	27.01	27.48	27.14	27.21	27.95	27.10
12	28.11	25.08	26.94	27.19	27.65	27.32	27.32	27.98	27.19
13	28.27	24.95	26.89	27.37	27.93	27.52	27.37	28.24	27.31
14	28.27	24.38	27.48	27.59	28.07	27.59	27.78	28.36	27.44
15	28.74	24.17	27.73	27.68	28.15	27.63	28.09	28.58	27.59
Mean	27.58	25.73	26.90	26.98	27.27	27.06	27.13	27.5	
LSD 0.05	Day= 1.09 ; treatment type= 0.62 ; Day * treatment type =1.31								

Figure.1 *Moringa oleifera* Callus Cultures Originated from Shoot Explants, Showing the Callus Mass Grown on MS Medium for Eight Weeks

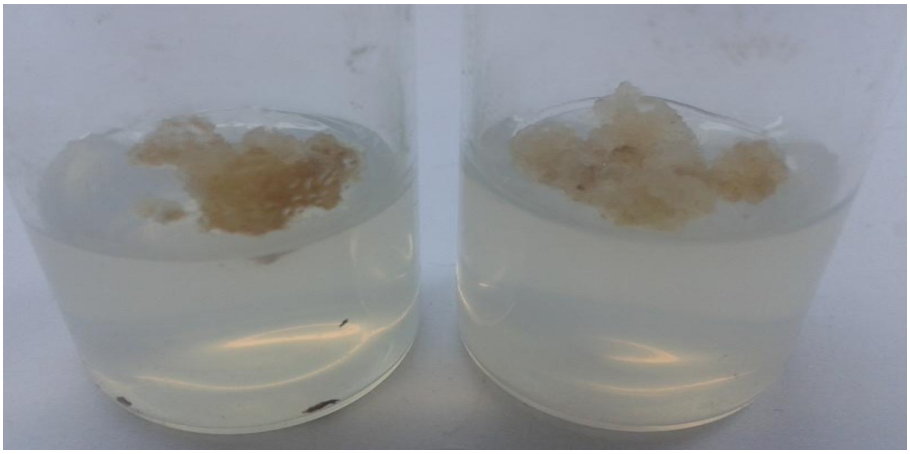

Table.4 Effect of Extract Doses on the Blood Glucose Level in Alloxan Induced Diabetic Albino Mice after 15 Days of Treatment, n=10

Type of Treatment / Days	mg/Ld + control	mg/Ld - control	Leaves extract			Callus extract			mg/Ld Mean
			20%	40%	80%	20%	40%	80%	
1	123.1	321	317.5	329.5	319.3	328.5	315.9	312.7	295.9
2	131.4	352.1	323.8	319.5	304.1	332.1	309.4	319.3	298.9
3	119.2	329.5	313.9	349.2	309.2	320.4	304.1	289.2	291.8
4	134.1	382.3	315.7	326.8	285.2	318.9	284.6	269.4	289.6
5	122.2	368.7	342.3	314.8	293.8	311.7	279.8	279.5	289.1
6	115.3	359.4	328.3	321.5	269.6	315.3	268.7	248.9	278.3
7	121.1	397.1	339.7	312.3	275.8	321.6	258.2	253.4	284.9
8	118.9	409.6	316.1	318.7	265.8	317.9	236.8	232.1	276.9
9	124.7	401.7	326	342.1	2598	312.6	239.5	229.6	279.5
10	109.6	423.6	348.2	338.2	276.8	308.7	227.4	223.8	282.3
11	121.5	452.8	329.8	313.8	231.3	317.9	224.1	205.3	274.5
12	119.8	509	353.9	312.8	238.9	313.5	221.6	178.2	280.9
13	118.4	493.5	323.1	309.4	226.9	303.2	213.1	152.3	267.4
14	132.7	501.7	319.5	298.7	221.7	285.1	207.9	131.9	262.4
15	117.3	513.4	326.1	277.4	216.2	262.4	211.8	113.3	254.7
Mean	121.9	414.3	328.2	318.9	266.2	311.3	253.5	229.2	
LSD 0.05	**Day= 21.96; treatment type= 18.53; Day * treatment type= 38.62**								

But callus extract was more effective than those of leave extracts in returning the animal's body weight and blood glucose level to the normal in comparison to negative control, using plant extracts for diabetic treatment appeared to be generally safe due to the presence of bioactive compounds especially in those of medicinal plants which have hypoglycemic effects through reduction insulin resistant, induction the releasing and inhibition glucagon secretion, slowing down the digestion and absorption of carbohydrates or by decreasing hepatic glucose production (Aja *et al.*,,2015). *M. oleifera* as a medicinal plant contains flavonoids, stilbeans, terpenoids, glycoside and alkaloids as its bioactive compounds as mentioned by Gupta and Misra, (2006), which also reported that the anti-diabetic effect of *M. oleifera* caused by an increase in insulin output or by inhibition of the intestinal absorption glucose. With the induction of callus cultures variable changes may occurred in the metabolic reactions of the growing cells and lead increase in their yield and activity

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