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Experimenting the possibility of callus development and growth from *Peganum harmala* L. leaf discs and assessment of the antibacterial activities of callus extract against *Salmonella* sp. and *Bacillus subtilis*

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Abstract

Peganum harmala L. (harmal) is a medicinal plant which has been used for ages in folk medicine for the treatment of many human diseases, including lumbago, asthma, colic, cancer, depression and malaria. Unfortunately, this valuable herb is facing the danger of extinction and decline due to climate change and human activities. So, introducing harmal to tissue culture systems might contribute to efforts of propagation, conservation and production of elite secondary metabolites needed for medicine. For this aim, this study was conducted to experiment the possibility of callus establishment in harmal from leaf discs to be used later on as a row material for *in vitro* micropropagation. Also the antibacterial activity of callus and wild plant extracts against two bacteria strains was assessed. Callus establishment was experimented by culturing young leaf discs on MS medium supplemented with various concentrations of 2,4-D (0.0, 0.2, 0.6, 1.2 and 2.0 mg/l), where full rate of callusing was obtained at level of 2.00 mg/ L. Moreover, best callus diameter (3.98 cm), fresh weight (3.79 mg) and dry weight (0.096 mg) were obtained in the MS medium plus 1.5 mg/L TDZ in combination with (2.00 mg/ L) 2,4- D. Meanwhile, the highest callus growth parameters were recorded in media supplemented with 0.1 M sucrose, while the lowest growth values were obtained in mannitol experiment at all levels. Moreover, the obtained results for antibacterial activity of callus cultures and wild plant extracts indicated that, both extracts were able to inhibit growth in both bacterial strains, but the wild type extract showed a stronger inhibitory effects on *Salmonella* sp. and *Bacillus subtilis* (inhibition zone: 1.6 and 1.8 cm, respectively) than those obtained from callus extract.

Key words: *Bacillus subtilis*, callus, *Peganum harmala*, plant growth regulators, *Salmonella* sp., sugar type.

Introduction

Peganum harmala L. (locally known as harmal) belongs to the family Zygophyllaceae which contains about 22 genera and more than 250 species [1]. *P. harmala* is a perennial herbaceous plant that is distributed in North Africa, Middle East, central Asia, India and Pakistan, as well as, South America and Mexico [2]. *P. harmala* is grown naturally in arid and semi-arid regions of Northern African and Asian deserts [3]. This plant is known as “Harmel” in North Africa, “Espand” in Iran, and “African Rue”, “Maxican Rue” or “Turkish Rue” in the United States [4]. It is used in folk medicine for the treatment of a variety of human diseases, including lumbago, asthma, colic, cancer, depression and malaria [4, 5]. Recently, a number of active alkaloids of *P. harmala* were identified, such as harmine, harmalol, harmin, quinazoline derivatives, vasicine and vasicinone, as they were extracted from different plant parts (leaves, stems, roots and seeds) before being used against a number of diseases [6, 7]. Also, it has been reported that the presence of such elite compounds in harmal has made this plant well known for its antibacterial, antifungal and antiviral activities [8]. Meanwhile, the short viability of harmal seeds in addition to climate change, drought, uncontrolled collection, urbanization and human activities are the most important challenges for propagation harmal, that would threaten the sustainability the natural supply of harmal elite secondary metabolites which are extensively needed for medicinal and research purposes [9, 10].

Plant tissue culture is important in terms of aseptic culture of cells, tissues, organs and their components under defined *in vitro* physical and chemical conditions. It is also an important tool for various applied studies and commercial applications [11].

Dedifferentiation of organized tissues into callus is a function of growth regulators, such as cytokinins and auxins, along with other components of the culture medium [12]. Meanwhile, defining the proper type and concentration of the growth regulators for each plant species is crucial for a successful callus growth and development [13]. Moreover, sugar type plays a significant role in growth of *in vitro* grown plants, and the impact of many carbohydrates on organs growth in addition to secondary metabolites production in these organs were extensively researched [14, 15].

So, due to the great medicinal importance of harmal in addition to the serious constrains that threaten its sustainability as a natural genetic resource, introducing harmal to tissue culture systems might contribute to the efforts of propagation, conservation and production of elite phytochemicals. For this aim, this study was conducted to experiment the possibility of setting a protocol for callus establishment and growth from leaf discs of harmal, to be used later on as a row material for *in vitro* propagation and secondary metabolite production purposes. Also, extracts from the *in vitro* grown callus and wild mother plant were assessed for their antibacterial activities against two bacterial strains, *Salmonella* sp. and *Bacillus subtilis*.

Materials and Methods

Establishment of plant material: Seeds of *P. harmala* were aseptically sterilized, cultured and incubated according to Zatimeh *et al.*[10]. After seed germination, germinated seedlings were transferred into hormone-free MS medium plus 0.1 M sucrose and subcultured for several intervals to obtain mother stock for the callus experimental part.

Callus establishment: For callus establishement leaves from *in vitro* grown microshoots were detached before being cut aseptically into discs. Next, the leaf discs were subcultured into callus establishment media consisting solid MS media [16] plus different levels of 2,4-dichlorophenoxy acetic acid (2,4-D) (0.2, 0.6, 1.2, 1.5 mg/L) and 1.5 mg/L TDZ and maintained under complete dark condition for 8 weeks. Data was collected for percentages of callus development at margins of the leaf discs in each treatment.

Determination of plant growth regulators effect of on callus growth: Callus cultures were transferred into a hormone free MS medium (HF-MS) for four days to remove any carry-over effect of plant hormones. To study the effect of plant growth regulators on callus growth and development, callus clumps were cut into pieces and each calli weighted 0.50 g was inoculated into MS media plus (2.0 mg/L) 2,4-D in combination with different levels (0.5, 1.0, 1.5 or 2.5 mg/L) of kinetin, BA, 2iP or TDZ, while control treatment consisted of hormone free MS media plus 0.1 M sucrose. Data was obtained after 8 weeks for callus diameter in addition to fresh and dry weights.

Determination of sugar type effect on callus growth: To study the effect of sugar type and level on callus growth, calli pieces (each piece weighted 0.50 g) were inoculated into callus maintenance media consisted of MS media plus the hormone combination that resulted the maximum callus growth parameters in 2.0 mg/L 2,4-D + 1.5 mg/L TDZ addition to different levels (0.1, 0.2, 0.3, 0.4 M) of sucrose, fructose, glucose, sorbitol and mannitol. The control media was MS media plus 0.1 M sucrose + (2.0 mg/L 2,4-D + 1.5 mg/L TDZ). Data was obtained after 8 weeks for callus diameter in addition to fresh and dry weights.

Assessment of antibacterial activity of callus and wild plant extracts against Salmonella sp.* and *Bacillus subtilis:

Preparation of extract: Samples from *in vitro* grown callus cultures of *Peganum harmala* and addition to leaves from harmal plants collected from the wild (wild mother plant) were dried and ground. For extract preparation, 10 g from each plant material was obtained after being dissolved in 100 mL of methanol and incubated for 72 hours. After incubation, the resulted extract from each plant material was filtered, and the extracts were evaporated to dryness by rotary evaporator at approximately 50°C. Each extract was dissolved in (100 mg/ml) DMSO (dimethylsulfoxide) and kept at -20°C.

Agar diffusion assay: Antibacterial activity was determined against two bacterial strains, *Salmonella* sp. and *Bacillus subtilis,* using agar diffusion assay. Three 6 mm diameter holes were made in Muller-Hinton agar plates containing bacterial strains. Then, 10 mg of the extracts were poured into the wells. Standard antibiotic (tetracycline 10 mg/mL) was used as reference or positive control. The plates were then incubated at 37°C for 24 h. Antibacterial activity was recorded by measuring zone of inhibition.

Experimental design: The arrangements of treatments in each experiment were according to the complete randomized design (CRD). Each tested concentration was replicated 4 times with 5 explants/replicate. The resulted data was analyzed according to the analysis of variance (ANOVA) using statistical package for the social sciences (SPSS) analysis system (version 17.0, http// www. winwrap.com), and means were separated according to the Tukey's HSD at probability level of 0.05.

Results and Discussion

Callus establishment: The results for callus establishment showed that, callus was not developed at margins of leaf discs when grown in the hormone free MS media (C1) (Table 1, Fig. 1). Meanwhile, adding 1.5 mg/ L TDZ to the media (C2) improved callus establishment rate (35%), but the callusing rate became higher when 2,4-D was added in combination with 1.5 mg/L TDZ to reach a maximum level (100%) at 2.0 mg/L 2,4- D (Table 1). This would agree with Al-Kiyyam [17] who reported that TDZ was the best growth regulator for callus induction from leaf discs of *Solanum nigrum* L.

Table 1. Effect of 2,4-D concentration on callus establishment rate from leaf discs of *Peganum harmala* L.

2,4-D concentration (mg/L)	Callus establishment rate (%)
C1	0.0 ± 0.0[Z]
0.1	35 ± 2.25
0.2	40 ± 1.95
0.6	45± 1.33
1.2	70 ± 1.82
2	100 ± 0.0

C1 treatment consisted of hormone free MS solid media. Percentages represent means± standard error.

Effect of plant growth regulators on callus growth and development: Adding growth regulators to the culture media improved the tested callus growth parameters compared to the control and at all levels (Table 2). In BA experiment, adding 1.00 mg/ L BA resulted in the highest callus diameter (2.88 cm) in addition to the maximum fresh and dry weights (2.44 and 0.053 g), while growth minimum values were recorded in the control (Table 2). Similar trend was also reported by Al- Kiyyam [17] who reported

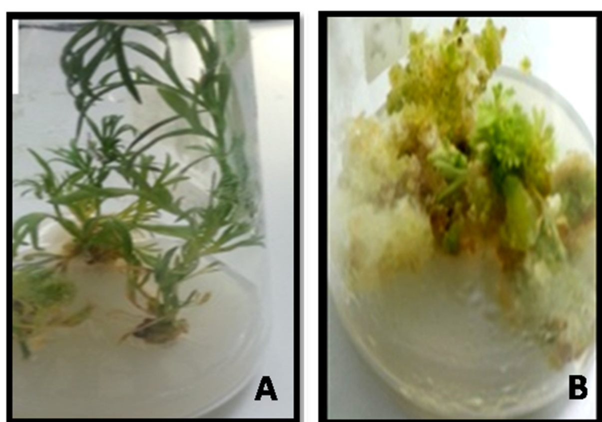

Figure 1. A: *In vitro* grown microshoots of *Peganum harmala* L. (Zatimeh *et al*. [10]). B: Callus establishment from leaf disc cultures grown in solid MS media + 2.0 mg/ L 2,4- D.

that, maximum callus growth was obtained in *Solanum nigrum* L. cultures when grown in MS media supplemented with 1.0 mg/L BA. Also the results for BA in our study agreed with Sheeba *et al.*[18] findings on callus induction and multiplication in *Physalis minima*. Additionally callus growth was reported to be successful when BA was applied to the culturing media in *Achillea fragrantissima* L. [19].

Moreover, data obtained in kinetin experiment revealed that, increasing kinetin concentration in the media increased significantly all growth parameters to reach maximum values for diameter, fresh and dry weights (3.64 cm, 2.88 and 0.067 g, respectively) at 1.50 mg/L kinetin (Table 2). This trend agrees with results reported by Raj *et al.* [20] and Slazak *et al.* [21], as callus multiplication in *Securinega suffruticosa* and *Viola uliginosa*, was successful when kinetin was applied to the media in combination with 2,4-D.

Moreover, TDZ results in the current study were most encouraging, and the highest diameter and fresh and dry weights (3.98 cm, 2.79 and 0.096 g) were recorded at 1.50 mg/ L TDZ (Table 2). TDZ was also reported to be effective in promoting callus growth in *Stephania tetrandra* and *Solanum nigrum* L. when used in combination with 2,4-D [17, 22]. Meanwhile, the performance of 2iP as a callusing promoter was poor compared to the other growth regulators used in the study, and the maximum growth parameters were recorded at 2iP concentration of 1.0 mg/L (Table 2). Poor callusing in *A. andrachne* was reported by Bertsouklis and Papafotiou [23], when low levels of 2iP were used, while increasing 2iP up to 5.0 mg/L 2iP was more effective for callusing. However, the data in our study revealed that TDZ was the best performing growth regulator when compared to others, as the maximum callus growth results were obtained when TDZ was added to the media at all levels (Table 2).

Effect of plant sugar type on callus growth and development: The results for the measured growth parameters revealed a clear influence of type of sugar added to the growth media on callus growth (Table 3). For example, callus growth parameters were highest when sucrose was used as carbon source at all levels followed by glucose compared to the values obtained in the other sugar types when added at similar concentrations (Table 3). This agrees with Praveena and Veeresham [24], who found that maximum biomass production in *Toddalia asiatica* was obtained in sucrose, followed by maltose, glucose and fructose. On the other hand promoting callus growth was most successful in (*Gossypium hirsutum* L.) when glucose was used as a carbon source compared to the other sugar types [25], which indicated that tissue cultured plants differ in their growth responses according to sugar type.

Table 2. Effect of different growth regulators types and concentrations in combination with 2.0 mg/ L 2,4-D on callus growth parameters in *Peganum harmala* L.

Growth regulator (mg/ L)		Callus diameter (cm)	Fresh weight (g)	Dry weight (g)
BA	0.0*	1.00±0.06^{Z} b	0.68±0.07 c	0.008±0.01c
	0.5	2.75 ± 0.02 a	1.81 ± 0.02b	0.026 ± 0.02 b
	1.0	2.88 ± 0.15a	2.44 ± 0.03 a	0.053 ± 0.03 a
	1.5	2.84 ± 0.03 a	2.37 ± 0.03a	0.045 ± 0.02 a
Kin	0.0*	1.00±0.06 b	0.68±0.07 b	0.008±0.01 b
	0.5	3.20 ± 0.10 a	2.79 ± 0.02 a	0.063 ± 0.01a
	1.0	3.27 ± 0.12a	2.85 ± 0.03 a	0.067 ± 0.01 a
	1.5	3.64 ± 0.90 a	2.88 ± 0.01 a	0.067 ± 0.03 a
TDZ	0.0*	1.00±0.06 b	0.68±0.07 d	0.008±0.01 c
	0.5	3.56 ± 0.06 a	2.94 ± 0.02 c	0.068 ± 0.02b
	1.0	3.86 ± 0.04 a	3.33 ± 0.04 b	0.072 ± 0.02 b
	1.5	3.98 ± 0.03 a	3.79 ± 0.03 a	0.096 ± 0.01 a
2ip	0.0*	1.00±0.06 c	0.68±0.07 c	0.008±0.01 c
	0.5	1.97 ± 0.01 b	2.59 ± 0.02 b	0.055 ± 0.02 b
	1.0	2.28 ± 0.03a	3.27 ± 0.01 a	0.070 ± 0.02 a
	1.5	1.89 ± 0.03b	2.56 ± 0.01 b	0.049 ± 0.03 b

*Control treatment consisted of hormone free MS solid media. ^{Z}Means within columns for each growth regulator having different letters are significantly different according to Tukey HSD at P≤0.05.

Moreover, our results showed that adding 0.1 M sucrose (control) to the culture media resulted in maximum callus diameter (3.98 cm) in addition to highest fresh and dry weights (3.79 and 0.96 g) (Table 3). Similar response was also obtained when sucrose level was doubled to 0.2 M (Table 3), as this increase in sugar concentration was translated into high values for callus diameter, in addition to fresh and dry weights (Table 3). However, the negative impacts of increasing sucrose level in the media became obvious at 0.3 and 0.4 M levels as all measured parameters were adversely affected and reached minimum values at 0.4 M sucrose (Table 3). This could be referred to the fact that, increasing sucrose concentration in the media was always confounded with a built up of osmotic stress [26].

In fructose experiment, the maximum callus diameter (2.54 cm) and fresh and dry weights (2.33 and 0.39 g) were obtained at level of 0.1 M, but these values were lower than those recorded in the control (0.1 M sucrose) (Table 3). Meanwhile, increasing fructose concentration to higher levels resulted in decreasing callus growth responses to reach minimum values at 0.4 M fructose (Table 3). Similar trend was also obtained in the rest of the experimented sugar types, but the decline in growth was most sever in mannitol experiment and at all levels (Table 3). Mannitol was repeatedly reported to be toxic on plant cells and increasing its levels beyond 0.3 M was also reported to be lethal on plants [27].

Table 3. Effect of sugar type and concentration on callus growth parameters in *Peganum harmala* L.

Carbon source (M)		Callus diameter (cm)	Fresh weight (g)	Dry weight (g)
Sucrose	Control (0.1)	3.98 ± 0.03 a	3.79 ± 0.03 a	0.096 ± 0.01 a
	0.2	2.73 ± 0.02 a	2.63 ± 0.02 a	0.082 ± 0.02 a
	0.3	1.97 ± 0.02 b	2.15 ± 0.04 c	0.038 ± 0.03bc
	0.4	1.82 ± 0.01 b	2.03 ± 0.03 c	0.020 ± 0.02 c
Fructose	Control	3.98 ± 0.03 a	3.79 ± 0.03 a	0.096 ± 0.01 a
	0.1	2.54 ± 0.03 b	2.33± 0.05 b	0.039 ± 0.02 b
	0.2	1.89 ± 0.03 c	2.00 ± 0.02 c	0.025 ± 0.02 bc
	0.3	1.69 ± 0.03c	1.83 ± 0.01c	0.020 ± 0.01bc
	0.4	1.21 ± 0.02d	1.27 ± 0.03 d	0.011 ± 0.02 c
Sorbitol	Control	3.98 ± 0.03 a	3.79 ± 0.03 a	0.096 ± 0.01 a
	0.1	1.93 ± 0.07 b	2.05 ± 0.01b	0.027 ± 0.04 b
	0.2	1.27 ± 0.02c	1.14 ± 0.05c	0.003 ± 0.01c
	0.3	1.14 ± 0.05d	1.09 ± 0.02c	0.001 ± 0.03 c
	0.4	1.08 ± 0.03d	1.05± 0.03c	0.001 ± 0.02 c
Glucose	Control	3.98 ± 0.03 a	3.79 ± 0.03a	0.096 ± 0.01 a
	0.1	2.68± 0.03 b	3.33± 0.01b	0.071± 0.03 b
	0.2	2.41 ± 0.01 b	2.19 ± 0.02 c	0.041 ± 0.01 c
	0.3	1.95 ± 0.02 cd	2.07 ± 0.03 cd	0.032 ± 0.03c
	0.4	1.80 ± 0.03 d	2.00 ± 0.03d	0.026 ± 0.02 c
Mannitol	Control	3.98 ± 0.03 a	3.79 ± 0.03 a	0.096 ± 0.01 a
	0.1	1.80 ± 0.03 b	2.18 ± 0.02 b	0.039 ± 0.02b
	0.2	1.31 ± 0.02 c	1.19 ± 0.02 c	0.006 ± 0.01 c
	0.3	0.75 ± 0.01 de	0.67 ± 0.01 d	0.004± 0.03 d
	0.4	0.5 3 ± 0.01 e	0.55 ± 0.03 d	0.002 ± 0.02 d

*Control treatment represent callus growth media which consisted of MS solid media plus 2 mg/ L 2,4-D + 1.5 mg/ L TDZ + 0.1 M sucrose. [Z]Means within columns for each sugar type having different letters are significantly different according to Tukey HSD at P≤0.05.

Generally, sugar type and level were reported to have an important role in growth and development of the *in vitro* grown tissues [28]. However, if sugars were added at elevated levels to the culture medium, these sugars might serve as osmotic stressors rather than energy sources as they would reduce water availability to the explants [26], and this would inhibit cell elongation and division[28], which was very clear in values recorded for callus growth parameters in *Peganum harmala* L..

Assessment of antibacterial activity of callus cultures and wild plant extracts: The antibacterial activity of callus cultures and wild plant extracts indicated that, both extracts were able to inhibit growth in both bacteria strains (Table 4). Meanwhile, the wild type extract showed stronger inhibitory effects on *Salmonella* sp. and *Bacillus subtilis* (inhibition zone: 1.6 and 1.8 cm, respectively) than those obtained from callus extract (Table 4, Fig. 2). This could be due to the presence of higher concentrations of active ingredient in the wild plant. Growth in the wild was usually reported to be combined with low water availability due to drought stress where plant cells tend to produce and accumulate secondary metabolites including alkaloids and tannins instead of cell division to prevent cell dehydration [27, 29]. On the other hand, under *in vitro* conditions optimum amounts of water are supplemented to callus cultures where the cells are directed to continue cell division due to the presence of growth regulators in the media rather than secondary metabolites production and accumulation [27]. Meanwhile, in our study results of the antibacterial activities of wild plant and callus extracts are promising, which agrees with many other related studies about harmal plants collected from the wild [30, 31]. However, more research is still needed on applying tissue culture techniques on callus cultures to direct cells into production of secondary metabolites including alkaloids rather than cell division.

Table 4. Antibacterial activity of callus cultures and wild plant extracts against *Salmonella* sp. and *Bacillus subtilis.*

Bacterial species	Zone of inhibition (cm)		
	Tetracycline	*Peganum harmala* (Wild type)	Callus extract (*In vitro*)
Salmonella sp.	2.4	1.6	1.1
Bacillus subtilis	2.3	1.8	1.2

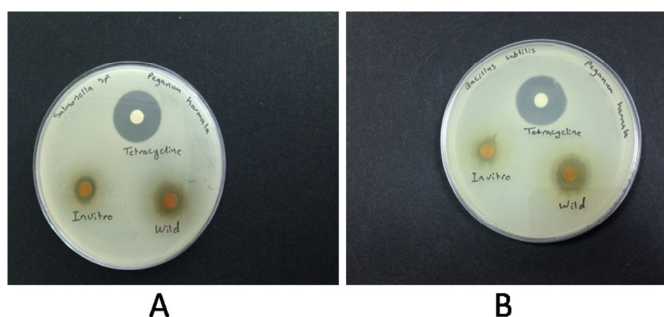

Figure 2. Inhibition zones resulted due to application of *Peganum harmala* L. callus cultures and wild plant extracts on A: *Salmonella* sp. and B: *Bacillus subtilis*.

Conclusions

Callus was successfully established in *Peganum harmala* L. leaf discs when grown in MS media supplemented with 2.0 mg/L 2,4-D. Callus growth was affected by type and concentration of the added growth regulator and sugar, as callus growth was maximized when cultured in MS media plus a hormonal combination of 2.0 mg/L 2,4-D + 1.5 mg/L TDZ in addition to 0.1 M sucrose. Moreover, results of the antibacterial activities of wild plant and callus extracts were promising, but more research is still needed on applying tissue culture techniques on callus cultures to direct cells into production of secondary metabolites including alkaloids rather than cell division, which would hopefully open the gate for using callus later on as a raw material for secondary metabolite production purposes.

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