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Research Article

IN- VITRO CULTURE OF *ARTEMISIA AUCHERI* BOISS ON FOUR DIFFERENT TISSUE CULTURE MEDIA FOR COMPARATIVE CYTOTOXIC EFFECT SAND GROWTH

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ABSTRACT

Objective: The extracts of *Artemiaia aucheri* have been reported to have anti-leishmania and cytotoxic effects. These activities depend on secondary metabolites. Plant tissue culture has been used as an alternative method for increasing the production of secondary metabolites. The present study was carried out to initiate callus cultures from *A. aucheri* and compare the effects of four different culture media on growth and cytotoxic production of *Artemisia aucheri* in callus culture after 20th subculture. Methods: Callus induction was initiated from seedlings on a Murashige and Skoog (MS) basal medium containing different concentrations of vitamins and combinations of growth regulators. Cytotoxicity of methanolic extract of callus culture grown on different culture media was assessed using the brine shrimp assay. Results: Different concentrations and combinations of phytohormones had significant influence only on growth index. Methanolic extract of callus culture on MS medium containing 6-benzylaminopurine 3 mg/l (BAP), naphthalene acetic acid 0.5 mg/l (NAA) and thiamine-HCl 0.5 mg/l was found to have the cytotoxicity (LC_{50}< 1000µg/ml) using brine shrimp lethality assay. However, methanolic extracts of callus culture on MS medium containing kinetin (Kin), indole-3-acetic (IAA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) had no cytotoxic effect. Conclusion: Among the four methanolic extracts by callus of *A. aucheri*, the best medium in supporting the cytotoxic production was MS supplemented with high level of BAP, NAA, and thiamine-HCl. This is the first report oncytotoxic effects of isolated culture of *Artemisia aucheri*.

Keywords:*Artemisia aucheri*, *Artemia franciscana*, Cytotoxic, Tissue culture, Growth index

INTRODUCTION

For centuries, mankind has been totally dependent on plants as source of carbohydrates, proteins and fats for food. In addition, plants have been a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. Over 80% of the approximately 30,000 known natural products are of plant origin [1-3].

Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites [4-11]. Christen et al. [12] reported for the first time the production of taxol (plaxitaxol) by *Taxus*cell cultures. Fett-Neto et al. [13] studied the effect of nutrients and other factors on plaxitaxol production by *T. cuspidata*cell cultures (0.02% yield on dry weight basis). Production of morphine and codeine in morphologically undifferentiated cultures of *Papaversomniferum* has been reported [14,15]. Ginseng has been recognized as a miraculous promoter of health and longevity and Ginsenoside Rg1 is one of the major active molecules from *Panaxginseng*[16].Chang and Hsing[17] obtained repeatable precocious flowering in the embryos derived from mature ginseng root callus cultured on a chemically defined medium. Berberine is an isoquinoline alkaloid which has been identified from a number of cell cultures, notably those of *Coptis*japonica [18], *Thalictrum*spp.[19,20], and *Berberis*spp.[21]. Tal et al. [22] reported on the use of cell cultures of *Dioscoreadeltoidea*for production of diosgenin,a precursor for the chemical synthesis of steroidal drugs which is tremendously important to the pharmaceutical industry [23]. Shikonin and rosmarinic acid are produed by cell suspension cultures of *Lithospermumerythrorhizon*[24]and cell cultures of *Coleus bluemii*[25], respectively. The synthesis of artemisinin has been studied in suspension cell [26], callus [27- 28], shoot [29-31], and hairy root cultures of *Artemisia annua*L.[32-34].

The genus *Artemisia*, belonging to the Asteraceae family, is a small herb and shrub which is represented by 34 species growing in different parts of Iran [35].Several isolated compounds from these species have been shown to have antimalarial, antibacterial, anti-inflammatory, plant growth regulatory, and cytotoxic (antitumor) activities [36]. The crude extract of *Artemisia vulgaris* L. has been used as an antimalarial agent for thousands of years. Sun et al. [37]found that artemisinin extracted from *A. vulgaris* had antitumor activity. *Artemisia aucheri* extract showed high cytotoxicity (LC_{50}< 35 µg/ml) on the brine shrimp assay [38]. *Artemisia aucheri* produces a portfolio of bioactive compounds including verbenone, camphor, 1, 8-cineole,*trans*-verbenol, Chrysanthenone,Mesitylene, α-pinene, acyclic monoterpenes, and monoterpenehydroperoxides [39-41]. Hence the present study was planned to investigate the strategies for producing substances with cytotoxic effectsusing plant tissue culture techniques.This is the first studyon the cytotoxic effectof isolated culture of *Artemisia aucheri*.

MATERIALS AND METHODS

Disinfection method

A.aucheri seeds were purchased from Pakanbazr Isfahan co. The seeds of *Artemisia aucheri* were washed with mild detergent in water, rinsed with running tap water for 1h and surface sterilized by immersingin 70% ethanol for 1 min. The seeds were then rinsed with autoclaved water before they were dipped into 1.5% sodium hypochlorite for 10 min. Afterwards, the surface-sterilized seeds were washed with autoclaved water three times. Ten seeds were placed on Murashige and Skoog (MS) [42] medium without any growth regulators.

Protocol for callus induction

One-month-old *in vitro* germinated seedlings of *Artemisia aucheri* were used as a source of explants for initiation of callus cultures. The media employed were (a) MS supplemented with Kin 2mg/l, IAA 1 mg/l, and 2, 4-D 0.1mg/l, (b) MS supplemented with Kin 2mg/l, IAA 0.1 mg/l, and 2, 4-D 1mg/l, (c) MS medium composed of a combination of BAP 2 mg/l, NAA 0.1mg/l, and thiamine-HCl 0.2 mg/l, and (d) MS medium composed of a combination of BAP 3 mg/l, NAA 0.5mg/l, and thiamine-HCl 0.5 mg/l. The pH was adjusted to 5.8 before sterilization by autoclaving at 121°C for 20 min. All media

contained 3% sucrose and 8% agar and incubated at 28 ± 1°C under completely dark condition. For growth and maintaining of callus stock, calli were subcultured on fresh media for 20 times every month.

Growth measurement

Growth of calli was determined by fresh and dry weight measurement. Callus growth represented by growth index, was calculated according to the following equation:

$$\text{Growth Index} = \frac{\text{Final callus fresh weight} - \text{Initial callus fresh weight}}{\text{Initial callus fresh weight}}$$

Dry Matter Content (%)

The fresh calli were dried at 60°C for 48 h and the dry matter content was estimated according to the following equation:

Callus Dry Matter (%) = Callus dry weight ×100 / Callus fresh weight

The experiments on calli were conducted with a minimum of five replicates. The data were analyzed by mean ± standard error followed by comparison of the means with the Duncan's test at $P<0.05$.

Preparation of callus extracts

After 20 subcultures, callus tissues were air-dried at 60°C and extracted by methanol for 72h at room temperature (27 ± 2.0 °C). The extracts were filtered and then the solvents were dried by vacuum rotary evaporator to obtain crude methanolic extract and stored at 4°C for further usage.

Cytotoxicity assays

The extract acute toxicity (mean lethal concentration) (LC_{50}) was evaluated using the brine shrimp (*Artemia franciscana*) assay as described elsewhere [43,44]. Briefly, 10 mg of dried brine shrimp eggs (Advanced Hatchery Technology, INC, USA) were deposited in fresh filtered seawater and allowed to hatch for 24 h. The assay was performed dissolving the extract of *A. aucheri.* Ten larvae were put in 10 ml seawater containing the extract of *A. aucheri* at concentrations between 30 and 2000 µg/ml. The LC_{50} assay was done using three replicates for each concentration, and counting of dead organisms was carried out 24 h after exposure to the different extract concentrations. All the experiments were conducted by quadruplicates and LC_{50} values as well as 95% confidence intervals were calculated using the probit method [45].

RESULTS

The growth characteristics and pattern of calli derived from seedling explants of *A.aucheri*growing on MS media with different concentration and combinations of phytohormones such as Kin, BAP , NAA, IAA, and 2, 4-D supplemented with various concentrations of vitamins were investigated (Table 1).

Table 1: MS media supplemented with different growth regulators and vitamin concentrations used for *A.aucheri* callus cultures

Media Codes	Basal Medium	Pyridoxine-HCl (mg/l)	Nicotinic acid (mg/l)	Thiamine-HCl (mg/l)	IAA (mg/l)	NAA (mg/l)	BAP (mg/l)	Kin (mg/l)	2, 4-D (mg/l)
I	MS	0.5	0.5	0.1	1.0	0.0	0.0	2.0	0.1
II	MS	0.5	0.5	0.1	0.1	0.0	0.0	2.0	1.0
III	MS	0.5	0.5	0.2	0.0	0.1	2.0	0.0	0.0
IV	MS	0.5	0.5	0.5	0.0	0.5	3.0	0.0	0.0

Calli growth patterns were expressed as callus fresh weight (mg), callus dry weight (mg), percentage of callus dry matter content, and growth index.The effects of four MS media under study on callus fresh weight, callus dry weight, and percentage of callus dry matter content did not differ significantly. The lowest growth index was obtained on MS medium supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 0.5 mg/l thiamine-HCl(1.94 ± 0.12). Moderate growth index was observed at concentration of 2 mg/l BAP, 0.1 mg/l NAA, and 0.2 mg/l thiamine-HCl(3.11 ± 0.23). The highest growth index was obtained on MS medium containing Kin (2 mg/l), IAA (1, mg/l), and 2, 4-D (0.1mg/l) and MS medium containing Kin (2 mg/l), IAA (0.1, mg/l), and 2, 4-D (1mg/l) which were 4.31 ± 0.56 and 3.81± 0.49,respectively (Table 2).

Table 2: Effects of plant growth regulators on callus fresh weight (mg), callus dry weight (mg), callus dry matter (%), and growth index of*A.aucheri*

Culture media	Fresh weight (mg)	Dry weight (mg)	Dry matter content (%)	Growth index
MS+2 mg/l Kin+1mg/l IAA+0.1mg/l 2, 4-D	1602.4±261.0	145.4 ± 12.3	6.15±0.14	4.31±0.56[b]
MS+2 mg/l Kin+0.1mg/l IAA+1mg/l 2, 4-D	1964.0±440.9	105.8±17.1	6.36±0.40	3.81±0.49[b]
MS+2 mg/l BAP+0.1mg/l NAA+0.2mg/l thiamine-HCl	1295.0 ± 200.0	148.4±30.6	5.78±0.29	3.11±0.23[ab]
MS+3 mg/l BAP+0.5mg/l NAA+0.5mg/l thiamine-HCl	927.0 ± 168.0	90.5±10.5	6.25±0.04	1.94±0.12[a]

Each numerical value represents the mean and standard error from 4 replications after 33 days of cultures. The different letters within the column indicate that the values are significantly different at $P<0.05$, following Duncan's multiple range tests.

MS medium supplemented with various concentration of IAA (1, 0.1 mg/l), Kin (2 mg/l), and 2, 4-D (0.1, 1 mg/l) led the callus to turn creamy and fragile. Friable creamy-white callus of *A.aucheri was* grown on MS medium supplemented with different concentration of

BAP (2, 3 mg/l), NAA (0.1, 0.5 mg/l), andthiamine-HCl (0.2, 0.5 mg/l). Calli grew well and remained proliferating even after 20 times of subculture.

Cytotoxicity of callus extracts

A total offour different isolated culturesof *A. aucheri* methanolic extracts were tested for their toxicity against brine shrimp using the brine shrimp lethality assay. Values varied between 30- 2000 µg/ml for 24 h exposure. The extracts of isolatedculture on MS medium supplemented with BAP (3 mg/l), NAA (0.5 mg/l), and thiamine-HCl (0.5 mg/l)showed significant cytotoxicity against brine shrimp (LC_{50}< 1000 µg/ml)(Fig 1).

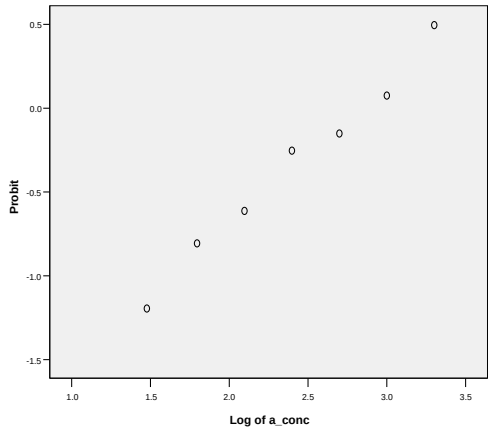

Fig 1. Regression line of *Artemia franciscana* mortality by callus extract of *A.aucheri*grown on MS medium containing 3.0 mg/l BAP + 0.5 mg/l NAA + 0.5 mg/l thiamine-HCl

The extract of isolatedculture on MS medium supplemented with BAP (2 mg/l), NAA (0.1 mg/l), and thiamine-HCl (0.2 mg/l)presented moderate cytotoxicity against brine shrimp; however, LC_{50} values were not observed(Fig 2).

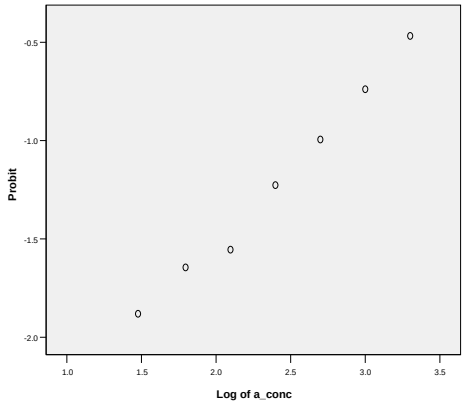

Fig 2. Regression line of *Artemia franciscana* mortality by callus extract of *A.aucheri* grown on MS medium containing 2.0 mg/l BAP + 0.1 mg/l NAA + 0.2 mg/l thiamine-HCl

The extracts of isolatedcultures on MS media containing various concentrations of IAA (1, 0.1 mg/l), Kin (2 mg/l), and 2, 4-D (0.1, 1 mg/l)had no cytotoxic effect (Figs 3 and 4).

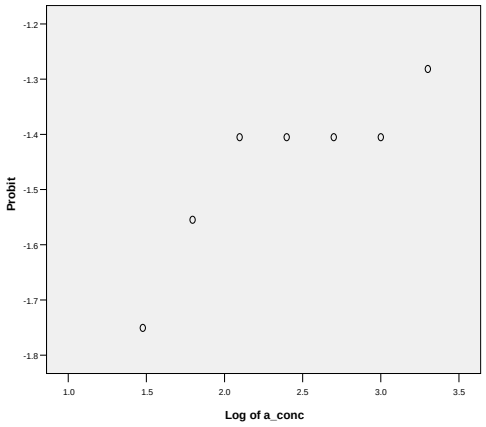

Fig 3. Regression line of *Artemia franciscana* mortality by callus extract of *A.aucheri* grown on MS medium containing 2 Kin mg/l + 1 mg/l IAA + 0.1 mg/l 2, 4-D

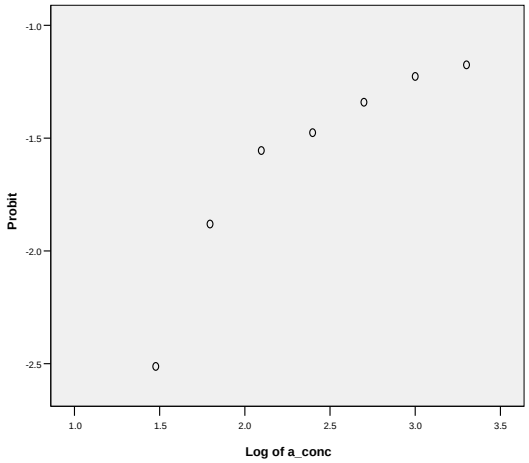

Fig 4. Regression line of *Artemia franciscana* mortality by callus extract of *A.aucheri* grown on MS medium containing 2 Kin mg/l + 0.1 mg/l IAA + 1 mg/l 2, 4-D

DISCUSSION

After 20th subculture, there were no clear differences in callus biomass (based on FW and DW measurement) among the treatments of the study. Similar results were also observed on callus production (based on FW and DW measurement) of *Ajugareptens* in tissue culture after 4th week subculture when used under light and light/dark cycling [46]. Also Akaneme and Ene-Obong [47] reported that there were no significant differences among the various combinations of NAA levels with Kin levels and NAA levels with BAP levels with respect to fresh weight production in callus of *Pinuscaribaea*.

Cancer is a big challenge to the world as effective remedy is very expensive and even impossible in some cases. Many scientists are now engaged to find potent remedy for cancer through the discovery of new and effective chemotherapeutic agents from plants and other sources [48].

Brine shrimp lethality assay is a primary assay to detect cytotoxic property of plant extract and further studies are required to establish the cytotoxicity of the plant extracts against human cancer cell lines. However, our results in the present study may predict which calli of *A. aucheri* will give better results on cancer cell lines.

Among the crude methanol extracts of the four different isolated cultures of *A. aucheri* examined in this study, one callus of *A.aucheri* resulted in significant cytotoxicity against brine shrimp. Despite no clear difference in the percentage of callus dry matter content in four different isolated culture of *A. aucheri*, there was noticeable difference between potent cytotoxic properties. Low and moderate growth index value could be the reason forthe production of bioactive compounds from *A. aucheri* cell culture. The extract of callus on MS medium supplemented with BAP (3 mg/l), NAA (0.5 mg/l) and thiamine-HCl (0.5 mg/l) in 20th passagescould be considered as potential sources of cytotoxic compounds. Similar result was also observed in production of equally increased concentration of bioactive compounds in isolated cultures of stem and leaf explants of *Justiciagendarussa*Burm. F. when compared to the plant samples [49].Gharehmatrossian et al [38] reported thatcallus extract of *A. aucheri* grown on MS medium supplemented with BAP (3 mg/l), NAA (0.5 mg/l), and thiamine-HCl (0.5 mg/l)did not show significant cytotoxicity against brine shrimpin 5th culture.This finding confirms also the observation of Hovhannisyan [50] for cytotoxic activity of callus extracts of *Nerium olender* who reported that the cytotoxic effect increased with callus subculturing from 4th to 14th passages.

MS media supplemented with BAP and NAA produced higher contents of cytotoxicity in comparison with MS media containing of Kin, IAA, and 2, 4-D in *A. aucheri* callus cultures. In contrast to the above mentioned result, Farouk et al [51] reported that MS media

supplemented with Kin and 2, 4-D produced higher contents of total phenolics, total flavonoids and antioxidant activities compared with the MS media supplemented with BAP and NAA in colocynthcallus cultures. Taniguchi *et al*. [52] reported that addition of BA (10 µM) and NAA (10 µM) to LS medium could enhance the production of triterpenes in callus cultures of *Eriobotrya japonica*. In addition, the shoot culture of *Menthaarvensiswas* reported toproduce terpenoid when the cultures were grownon MS medium supplemented with BA (5 mg/l) and NAA (0.5 mg/l) [53]. Similar observations have been reported for callus cultures of *Eucommiaulmoides*. Callus cultures of *E. ulmoides*showed high levels of accumulation of pinoresinol di-o- glucoside when 4 mg /l of BA and 3 mg/ l of NAA were added to the growth medium [54].

Since in most cases toxicity is associated with pharmacological properties, it was deduced that the extracts from isolatedcultures onMS media supplemented with different concentrations of BAP (2, 3 mg/l), NAA (0.1, 0.5 mg/l), andthiamine-HCl (0.2, 0.5 mg/l) had the best bioactivity.

Further investigations using single components from these extracts may explore potent cytotoxic properties.

CONCLUSION

The cell culture offers many advantages to scale-up production of secondary metabolites in plant cells of interest. In this study, we reported for the first time, a procedure for initiation and establishment of callus cultures of *A.aucheri*, which was able to accumulate high level of cytotoxic properties. The results demonstrated the importance of the components of growth regulators and thiamine-HCl in growth medium, in callus growth and cytotoxic production. These findings provide some basic information from the production of bioactive compounds from *A.aucheri* cell culture.

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