Tropane alkaloids of *Atropa belladonna* L.: *in vitro* production and pharmacological profile

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Objective
The aim of the present work was to study the production of tropane alkaloids by *in vitro* cultures of *Atropa belladonna* L. and to evaluate the anticonvulsant, antinociceptive, motor incoordination, and antioxidant activities of both *in vitro* and original plant extracts.

Background
A. belladonna is a very important medicinal plant with multipurpose therapeutic effects. The yield of its alkaloid content is very low, which makes it difficult for industrial application.

Materials and methods
Murashige and Skoog media were used for callus and plant differentiation induction from leaf explants of *A. belladonna* L. Qualitative and quantitative analysis of alkaloids was carried out using high-performance liquid chromatography. The anticonvulsant activity was screened by the pentylenetetrazole seizure test. The antinociceptive activity was evaluated by adopting the writhing test, whereas motor incoordination was evaluated using the rotarod test. In addition, antioxidant activity was estimated using the 2,2'-diphenyl-1-picrylhydrazyl radical-scavenging test.

Results
Callus and differentiated plants were successfully induced in Murashige and Skoog media supplemented with growth regulators. High-performance liquid chromatography analysis revealed the production of higher concentrations of tropane alkaloids in differentiated plants than in the original plant. Anticonvulsant and antinociceptive activities, motor incoordination, and the antioxidant effect of callus extracts were much higher than those of the original plant leaf extract.

Conclusion
Plant tissue culture could be considered as an efficient and alternative source of continuous supply of tropane alkaloids with potent anticonvulsant, antinociceptive, motor incoordination, and antioxidant activities. It is also a powerful tool for producing *A. belladonna* strain with a high tropane alkaloid content.

Keywords:
anticonvulsant, antinociceptive, motor incoordination, antioxidant, *Atropa belladonna* L., callus, differentiation, tropane alkaloids

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Introduction

Atropa belladonna L. belongs to the subtribe lyciinae (family Solanaceae) known as the deadly nightshade. It is a perennial plant native to central and southern Europe and cultivated worldwide. The plant is the most important source of tropane alkaloids in the family Solanaceae [1].

These tropane alkaloids include hyoscine (scopolamine), hyoscyamine, and its enantiomer atropine. Tropane alkaloids are of special interest, because of their therapeutic effects. *A. belladonna* L. has anticholinergic activity and sedative effect, and is used as an antispasmodic agent in bronchial cases, for cold and fever [2,3]. In addition, tropane alkaloids have mydriatic and analgesic properties [4] and anticonvulsant effects [5] in the treatment of Parkinson's disease and motion sickness [6].

Currently, there are many trials to produce materials of natural origin, not only by organic chemical synthesis, but also by biological methods. Because of the complex structure of these metabolites, their production remains unsuccessful on the indusial scale [2,7]. Consequently, commercial supply of *A. belladonna* L. is limited, because of the low abundance of tropane alkaloids in natural plants [1]. Although the biosynthesis of alkaloids is genetically controlled, it might be affected by different factors such as light, temperature, and fertilization [4]. There have been many attempts to enhance and improve the production of tropane alkaloids by transgenic cultures [1,2]. The use of γ-radiation and gibberellic acid for the production of mutant plants to achieve high alkaloid content is also reported [8,9].

Because of the therapeutic importance of tropane alkaloids and the difficulty to achieve a high yield

of these alkaloids, the aim of this work was the enhancement of tropane alkaloid production in both callus and differentiated plants and the comparison of the pharmacological activity of *in vitro* calli and original leaf plant extracts for their anticonvulsant, antinociceptive, motor incoordination, and antioxidant activities.

Materials and methods

Materials

Plant material

Leaves of *A. belladonna* L. obtained from the plant grown in the Medicinal Plant Farm of the College of Pharmacy, Cairo University, were identified by Dr Salwa Kawashty, Department of Phytochemistry and Plant Systematics, National Research Centre (NRC). A voucher sample was deposited at NRC Herbarium with the registration number M105.

Chemicals, standards, and drugs

Media components and growth regulators for *in vitro* cultures were tissue culture grade. Solvents for analysis were high-performance liquid chromatography (HPLC) grade. Standards for HPLC were hyoscine, hyoscyamine, and atropine, tween-80, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), and pentylenetetrazole (PTZ) (Sigma, St Louis, Missouri, USA), diphenylhydantoin sodium EL Nasr Pharm. Chem. Co., Abu Zaabal, Qaliubiya, Egypt, and atropine sulfate ADWIC pharmaceutical division, EL Nasr Pharm. Chem. Co., Abu Zabal, Egypt.

Animals

Mice were purchased from the Animal House Colony of the NRC, Cairo, Egypt, and were housed under standardized conditions (room temperature 23 ± 2°C; relative humidity 55 ± 5%; 12-h light/dark cycle). All animals were allowed free access to water and standard mice chow throughout the whole experimental period. Animal procedures were performed as per the Ethics Committee of the NRC and in accordance with the recommendations for the proper care and use of laboratory animals 'Canadian Council on Animal Care Guidelines, 1984'. After 7 days of acclimatization, the animals were randomly assigned to control, reference, and tested experimental groups of 6–9 mice each. All the test compounds were suspended in 7% Tween-80 saline solutions.

Methods

Callus induction and in vitro plant differentiation

A. belladonna L. leaves were washed with tap water, immersed in clorax 10% v/v (sodium hypochlorite) for 20 min, immersed in 70% ethyl alcohol for a few seconds, and then rinsed twice with sterile distilled water under sterile conditions in a laminar airflow cabinet. The leaves were sliced and aseptically cultured in glass jars containing Murashige and Skoog (MS) media [10], which contained 3% sucrose, and supplemented with different concentrations of growth regulators: Benzyl adenine (BA), kinetin (Kin), indole acetic acid (IAA), and naphthalene acetic acid (NAA). Cultures were maintained at 26 ± 2°C and 16/8 h photo period. Subculturing of calli was performed every month. Initiated shoots were transferred to basal media to allow root formation [11].

Extraction and analysis of the tropane alkaloids

The plant material was dried at a temperature not exceeding 40°C and ground. The dried powder (50 mg) was extracted with chloroform: methanol : 25% ammonia (15 : 15 : 1 v/v/v) [7]. Each of the dried extracts was dissolved in methanol, filtered through 0.45-Millipore filters, and analyzed using HPLC [4].

HPLC instrument Young Lin (Young Lin Cooperation, Seoul, South Korea) consists of a Reprosil-Pur Basic C18 5 μm (dimension: 250 × 4.6 mm) column (flow rate = 0.5 ml/min) and a UV detector (λ_{max} = 210 nm). The mobile phase used was an isocratic solution of water : Acetonitrile (65 : 35 v/v). Standard hyoscine, hyoscyamine, and atropine were dissolved in methanol at a concentration of 1000 ppm. Different concentrations of each standard were used and the calibration graphs were established by the plotting area under the peak of each standard against the corresponding concentration. Linear calibration of the alkaloids in each sample was calculated from linear regression equation of each standard.

The regression equation for hyoscine is as follows:

$Y = 1929.3X - 3248.9$, $R^2 = 0.9982$.

The regression equation for hyoscyamine is as follows:

$Y = 1866.2X - 6960.8$, $R^2 = 0.995$.

The regression equation for atropine is as follows:

$Y = 2733.9X - 928.76$, $R^2 = 0.9967$.

where Y is the peak area, X is the concentration, and R^2 is the correlation coefficient.

Pharmacological study

Evaluation of anticonvulsant activity

An aqueous solution of PTZ (85 mg/kg) [12] was administered in a loose fold of skin on the back of the mice

neck half an hour after intraperitoneal injection of the test extracts. The mice were observed during the 30 min after the injection of subcutaneous PTZ for the occurrence of seizures. A threshold convulsion was defined as one episode of clonic convulsions that persisted for at least a 5-s period. The absence of a single 5-s episode of clonic spasms during the period of observation was chosen as an index for the protective effect.

Antinociceptive activity

This activity was investigated by the writhing test [13]. Groups of six mice of both sexes (20–25 g) were used. One group, which served as the control, was injected with 0.1 ml of the vehicle. The test extracts were intraperitoneally administered in doses of 80 and 160 mg/kg, 30 min before intraperitoneal injection of freshly prepared acetic acid [2% (w/v) in saline; pH = 2.7, 10 ml/kg body weight]. The animals were then immediately placed individually into a transparent plastic box. The number of writhes, a response consisting of an abdominal wall, pelvic rotations, followed by hind limb extension, was counted during continuous observation for 20 min starting 5 min after acetic acid injection, and the percentage inhibition of writhing was expressed. Acetylsalicylic acid (200 mg/kg) was used as a reference drug against which the test extracts were compared.

Evaluation of motor coordination

The motor coordination of the animals was evaluated by adopting the rotarod test [14] (rotarod; UGO Basile, Varese, Italy). In this test, the animals were trained to maintain equilibrium on a rotating 1-inch-diameter knurled plastic rod for 120 s in each of three trials. Only animals that fulfilled this criterion were included in the experiment. The animals in the experimental groups (n = 6-9) were given an intraperitoneal injection of one of the test extracts at a dose of 80 and 160 mg/kg in 7% aqueous suspension of Tween-80. Thirty minutes later, the mice were placed again on the rotating rod at a speed of 16 rpm and the motor performance time was recorded up to 120 s. Animals were tested for their motor coordination capacity, which was indicated by the ability of the animal to maintain equilibrium on the rod for at least 120 s.

Antioxidant activity

The free-radical-scavenging capacity of tropane alkaloids of *A. belladonna* L. cultures and intact leaf plant extracts was determined using the stable free radical DPPH according to Brand-Williams *et al.* [15]. Freshly prepared methanolic DPPH solution (100 μmol/l) was added to 200 μg/ml of the methanolic extract in a 96-well microplate, and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm using a plate reader. The percentage of antioxidant activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the sample in the presence of the plant extract.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis of the obtained data was performed using one-way analysis of variance followed by a Student–Newman–Keuls post-hoc comparison. A result was considered statistically significant where P value was less than 0.05.

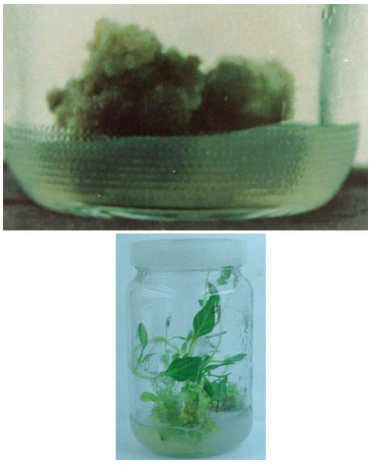
Results and discussion

In vitro callus initiation, differentiation, and tropane alkaloids analysis

The present results showed that calli were induced in leaf cultures on MS media with Kin, BA, and IAA at concentrations of 0.5, 0.5, and 2 mg/l, respectively (culture I), and in MS media supplemented with BA and NAA at concentrations of 0.5 and 1 mg/l (culture II). Differentiation was successfully induced in media containing BA and IAA at concentrations of 0.2 and 2 mg/l (Fig. 1). The role of growth regulators in callus induction and differentiation is well established [16].

HPLC analysis revealed the presence of tropane alkaloids: Hyoscine, hyoscyamine and its enantiomer atropine in calli, the differentiated plant, and the original mother plant (Fig. 2).

Figure 1



Callus (a) and differentiated plant of *Atropa belladonna* (b).

The results presented in Table 1 revealed that media containing a combination of Kin, BA, and IAA (culture I) yielded a higher alkaloid content than media containing Kin and NAA (culture II), in good agreement with literature indicating that active alkaloid metabolite production varied according to different media composition [17].

Furthermore, the results demonstrated that leaves of the differentiated plants contained a higher amount of tropane alkaloids (10.88 mg/g) than that of the two calli cultures (6.90, 2.98 mg), respectively, on a dry weight basis. This is compatible with previous studies reporting that the tropane alkaloid content increased in differentiated cultures of *Datura innoxia* [18]. Alkaloids were also biosynthesized in higher concentrations in regenerated shoots than in the *Solanum* spp. callus culture [19]. In addition, the present results clarified that the differentiated plant produced much higher alkaloids than the original plant, which is in good agreement with studies reporting that careful selection of productive cells and culture conditions resulted in the accumulation of secondary metabolites in higher concentrations than in original mother plants [19,20].

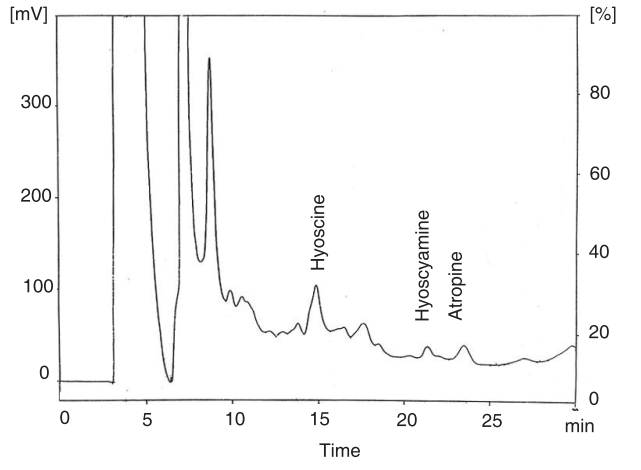
The present data illustrated that hyoscyamine and its enantiomer atropine are the predominant tropane alkaloids in the mother plant. Early studies showed that most members of the lyciinae produced alkaloids of the hyoscyamine type and that is the principal alkaloid of entire *A. belladonna* L. plants [21].

In contrast to the original mother plant, *in vitro* callus cultures and differentiated plants produced hyoscine in higher concentrations than hyoscyamine-type alkaloids. *In vitro* biotransformation through plant cell and tissue culture is a well-known process. In all, 10–20% of hyoscyamine added to the medium was converted to hyoscine within 24 days by root cultures of *Hyoscyamus niger* [22]. β-Methyldigitoxin was converted to β-methyldigoxin in *Digitalis lanata* cultures.

In vitro hyoscine production in differentiated leaves and culture I was much higher than in the original plant. The increment of hyoscine in differentiated leaves and culture I was 165.31 and 77.40%, respectively, more than its concentration in the original plant leaf extract. The results are confirmed by studies reporting that hyoscine was produced in higher concentrations in transgenic hairy root cultures of *A. belladonna* L. than in the original plant [1]. The amounts of hyoscine produced in our study from differentiated leaves and culture I were 6.10 and 4.08 mg/g, respectively, which is much higher than those obtained from the previous hairy root culture (2.2 mg/g) on a dry weight basis. It is worth mentioning that differentiated leaves produced tropane alkaloids in the same sequence as that of callus cultures with hyoscine as the major alkaloid, which reached 56.07% of the total tropane alkaloids produced.

In vitro total tropane alkaloids in the present study produced by differentiated leaves, callus of culture I, and callus of culture II (10.88, 6.9, and 2.98 mg/g), respectively, are much higher than total tropane alkaloids produced by calli cultures of mutant lines of *A. belladonna* L. (1.76 mg/g) [9].

Figure 2



High-performance liquid chromatography chromatogram of *in vitro* tropane alkaloids from *Atropa belladonna*.

Table 1 Tropane alkaloids of *in vitro* culture and original leaf extracts of *Atropa belladonna* L. (mg/g dry weight)

Extract materials	Hyoscine	Hyoscyamine	Atropine	Total tropane alkaloids
Leaves of original plant	2.30	3.05	3.35	8.70
Leaves of differentiated plant	6.10	4.19	0.59	10.88
Culture I	4.08	2.47	0.35	6.90
Culture II	1.40	1.33	0.25	2.98

Pharmacological studies

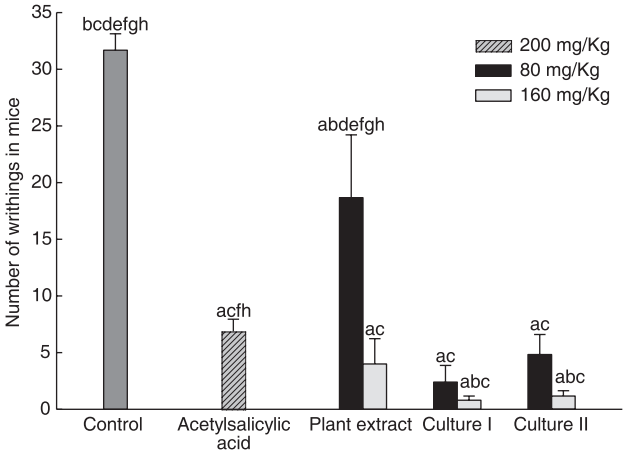
Regarding the anticonvulsant activity, our data showed that the original leaf extract, culture I, and culture II extracts exhibited anticonvulsant activities to variable degrees that was dose-dependent (Table 2). The present data are in agreement with studies reporting that tropane alkaloids exhibited anticonvulsant activity [5]. The activity was directly proportional to the percentage of hyoscine to total tropane alkaloid content, wherein the maximum protection of 67% was reached by the callus extract of culture I (160 mg/kg), which contained 59.13% hyoscine of the total tropane alkaloids content, followed by 50% protection by the callus extract of culture II (160 mg/kg), which contained 46.98% hyoscine compared with 33% protection of the leaf

extract of the original plant, which contained 26.43% hyoscine. In addition, culture I extract (80 mg/kg) exerted equipotent anticonvulsant activity as culture II (160 mg/kg). In contrast, atropine sulfate at the tested doses (80 and 160 mg/kg) exhibited 14 and 17% protection, respectively. Hyoscine is more preferred as a parasympatholytic agent than hyoscyamine-type tropane alkaloid, as the latter has a stimulant action on the central nervous system [22]. The results showed that the more the percentage of the hyoscyamine-type alkaloid in the total tropane alkaloid content, the lesser the anticonvulsant activity.

In the current study, the antinociceptive effect of culture I, culture II, and the original leaf plant extracts on the writhes induced by acetic acid was studied. Results illustrated in Figure 3 revealed that both culture I and culture II and the original leaf plant extracts exhibited significant antinociceptive activity compared with the control group. Moreover, culture extracts I and II at the dose level of 80 and 160 mg/kg exerted more potent antinociceptive activity than the original leaf plant extract (80 mg/kg). Meanwhile, the tested cultures at a dose of 160 mg/kg exerted a significant antinociceptive activity compared to acetylsalicylic acid (200 mg/kg) used as the reference drug. Furthermore, culture I and culture II exhibit equipotent antinociceptive activity at a dose level of 160 mg/kg. In contrast, the original leaf plant extract at a dose level of 160 mg/kg exhibited insignificant antinociceptive activity than that of acetylsalicylic acid (200 mg/kg) used as the reference drug. The present results support the finding that *A. belladonna* plant is used for cold and fever and have analgesic activity [3,4].

Concerning the motor coordination effect of the tested original leaf plant, culture I, and culture II extracts, the data presented in Table 3 revealed that the highest motor performance alteration was achieved by the culture I extract at a dose level of 160 mg/kg (83%); meanwhile, at the same dose level, culture II exhibited 50% alteration in motor incoordination compared with atropine sulfate (67%) used as the reference drug. In addition, both culture

Figure 3



Antinociceptive effects of tropane alkaloids of *Atropa belladonna* L. cultures and original leaf plant extracts adopting acetic acid-induced writhing in mice. Data are presented as mean±SEM (n = 6). The test extracts were intraperitoneally administered in doses of 80 and 160 mg/kg. The control group received 0.1 ml of the vehicle. Both the test extracts and the vehicle were injected 30 min before intraperitoneal injection of freshly prepared acetic acid [2% (w/v) in saline; pH = 2.7, 10 ml/kg body weight]. Acetylsalicylic acid (200 mg/kg) was used as a positive control. a, significantly different from the control group at $P < 0.05$; b, significantly different from acetylsalicylic acid at $P < 0.05$; c, significantly different from the plant extract (80 mg/kg) at $P < 0.05$; d, significantly different from the plant extract (160 mg/kg) at $P < 0.05$; e, significantly different from culture I (80 mg/kg) at $P < 0.05$; f, significantly different from culture I (160 mg/kg) at $P < 0.05$; g, significantly different from culture II (80 mg/kg) at $P < 0.05$; and h, significantly different from culture II (160 mg/kg) at $P < 0.05$.

Table 2 Anticonvulsant activity of tropane alkaloids of *Atropa belladonna* L. cultures and original leaf plant extracts against pentylenetetrazole-induced seizures in mice

Groups	Dose (mg/kg)	Protection[a] (%)	Hyoscine in tropane alkaloids (%)
Control	0	0	—
Diphenylhydantoin sodium	27.5	62.5	—
	55	100	—
Atropine sulfate	80	14	—
	160	17	—
Original leaf plant extract	80	17	26.43
	160	33.3	
Culture I	80	50	59.13
	160	67	
Culture II	80	43	46.98
	160	50	

[a]Data show the percentage protection against pentylenetetrazole (PTZ)-induced seizures in mice. Animals (n = 6–8) were subcutaneously injected with PTZ (85 mg/kg), 30 min after intraperitoneal injection of the test extracts; The mice were observed during the 30 min after the injection of subcutaneous PTZ for the occurrence of seizures.

Table 3 Effect of tropane alkaloids of *Atropa belladonna* L. cultures and original leaf plant extracts on motor coordination

Groups	Dose (mg/kg)	Number of animals exhibiting motor incoordination[a]	Motor incoordination[b] (%)
Atropine sulfate	80	3/6	50
	160	4/6	67
Original leaf plant extract	80	2/6	33
	160	2/6	33
Culture I	80	2/6	33
	160	5/6	83
Culture II	80	4/9	44
	160	3/6	50

[a]The data show the number of animals falling on the rotating rod (n = 6–9); [b]The data presented show the percentage of motor incoordination; Groups of animals (n = 6–9) were examined 30 min after intraperitoneal administration of one of the test extracts at a dose of 80 and 160 mg/kg; Standard atropine sulfate (80 and 160 mg/kg) was used as a reference drug; Thirty minutes after injection of the test extracts and reference, mice were placed on the rotating rod at a speed of 16 rpm and the motor performance time was recorded up to 120 s.

I and II extracts exhibited higher motor incoordination effects than the original leaf plant extract. It is worth mentioning that the presented data are in accordance with previous reported studies on tropane alkaloids in the treatment of Parkinson's disease and motion sickness [6].

The stable radical DPPH has been used widely for the *in vitro* assessment of antioxidant activity. The assay is based on the reduction of DPPH radicals in methanol on the addition of an antioxidant agent, which causes an absorbance decrease at 517 nm. The data revealed that the antioxidant activity of the tested extracts of culture I, culture II, and original leaf plant extracts at a concentration of 200 μg/ml exhibited a relatively moderate DPPH-scavenging activity of 39, 40, and 31%, respectively. In addition, the current result indicated that both culture extracts had better DPPH-scavenging activity than the original leaf extract. In conclusion, the pharmacological evaluation of the different tested extracts clearly showed that the culture extracts I and II possessed more potent anticonvulsant, antinociceptive, motor incoordination, and antioxidant activities compared with the original leaf plant extract.

Conclusion

The results obtained support the idea that the plant cell could act as a bioreactor for secondary metabolite formation. Tropane alkaloids could be biosynthesized in good concentration all over the year as compared with once or twice at the most from original plants. Plant tissue culture is also a powerful tool for producing *A. belladonna* strain with a high tropane alkaloid content. The predominance of hyoscine in the cultures is preferable, as hyoscyamine-type alkaloid has a central nervous system-stimulant effect. The use of culture extracts at concentrations lower than that of the standard drug and original plant extract to achieve the desired effect help to lower the side effects of tropane alkaloids.

The pharmacological evaluation of the extracts of the original leaf, culture I, and culture II clearly revealed that extracts of culture I and II possessed more potent anticonvulsant, antinociceptive, motor incoordination, and antioxidant activities compared with the original leaf plant extract.

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Conflicts of interest

There are no conflicts of interest.

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