

Anticonvulsant activity of *Dorema ammoniacum* gum: evidence for the involvement of benzodiazepines and opioid receptors

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Abstract

This study investigated the anticonvulsant activity and possible mechanism of action of an aqueous solution of *Dorema ammoniacum* gum (DAG) which has been used traditionally in the treatment of convulsions. In this study, the anticonvulsant activity of DAG was examined using the pentylenetetrazole (PTZ) model in mice. Thirty male albino mice were divided randomly and equally to 5 groups, and pretreated with normal saline, diazepam, or various doses of DAG (500, 700, and 1000 mg/kg, i.p.), prior to the injection of PTZ (60 mg/kg, i.p.). The latency and duration of seizures were recorded 30 min after PTZ injection. Pretreatments with naloxone and flumazenil in different groups were studied to further clarify the mechanisms of the anticonvulsant action. Phytochemical screening and thin layer chromatography (TLC) fingerprinting of ammoniacum gum was also determined. DAG showed significant anticonvulsant activity at all doses used. The gum delayed both the onset and the duration of seizures induced by PTZ. Treatment with flumazenil before DAG (700 mg/kg) inhibited the effect of gum on seizure duration and latency to some extent and administration of naloxone before DAG also significantly inhibited changes in latency and duration of seizure produced by DAG. The percentage inhibition was greater with naloxone than with flumazenil. This study showed that DAG had significant anticonvulsant activity in PTZ-induced seizures, and GABAergic and opioid systems may be involved. More studies are needed to further investigate its detailed mechanism.

Keywords: *Dorema ammoniacum*; Anticonvulsant; Flumazenil; Naloxane; Pentylenetetrazole

INTRODUCTION

Epilepsy is a common neurological disorder which is identified by recurrent spontaneous seizures and affects about 50 million people worldwide (1-4).

There is an increasing need to explore new natural medicines due to inadequacies of conventional treatments (5).

There are some herbs in Iranian traditional medicine that have been used for the treatment of epilepsy (6). *Dorema ammoniacum* gum (DAG), known as Oshagh, is recommended for treatment of seizures in Iranian traditional medicine (7,8). It is an oleo gum resin obtained from the stem and leaf of *Dorema ammoniacum* D. Don (Umbelliferae) (9). Genus *Dorema* exhibited six species in Iran,

among which two are endemic, namely, *D. ammoniacum* and *D. aucheri* Boiss(10).

D. ammoniacum grows to a height of 1-2 m in many arid and semi-arid regions of Iran, such as Yazd, Isfahan and Semnan(10,11). This plant produces a medicinal gum resin commonly known as *ammoniacum* gum which has medicinal and industrial applications (12).

According to our investigations, the antiepileptic effects of DAG have not previously been studied.

Therefore, in the present study, for the first time, the anticonvulsant activity and possible mechanisms of action of DAG were studied using pentylenetetrazole (PTZ)-induced seizures in rats.

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MATERIALS AND METHODS

Plant material

The DAG was purchased from a local herbal market in Tehran, Iran and its authenticity was approved by Dr. G. Amin, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. A voucher specimen of the plant was deposited in the herbarium of School of Pharmacy (DMP-819). The gum was dissolved in distilled water and the aqueous solution was used for the study.

Animals

One hundred and fourteen healthy male albino mice weighing 25-30 g were housed in the animal unit of Iran University of Medical Sciences under standard laboratory conditions (temperature 23 ± 2 °C) with 12-h dark and 12-h light cycle. The animals had free access to a standard dry pellet diet and tap water *ad libitum*. All possible steps were taken to avoid animal suffering at each stage of the experiments. All procedures involving animals were conducted according to the guidelines for the care and use of mammals in neuroscience and behavioral research (National Research Council 2003) and guideline of Institutional Animal Ethical Committee (IUMS-AC).

Preliminary phytochemical screening

Phytochemical investigation of DAG was carried out using standard methods and tests as published previously (13-15). The test for tannins was carried out by subjecting 1 g of extract in 2 mL of distilled water; the mixture was filtered and ferric chloride reagent was added to the filtrate. The gum was subjected to the "frothing" test for the identification of saponins and to fehling's test for glycosides. Alkaloids were detected in the alkaloid fraction obtained by a classical acid:base extraction procedure for alkaloids and analyzed by TLC using chloroform:methanol:ammonia solution 25% (8:2:0.5) as solvent system, spots were detected by spraying with Dragendorff's reagent. The presence of flavonoids was determined by adding 1% aluminum chloride solution to the

extract and examining for yellow coloration. In another test for flavonoids, diluted ammonia (5 mL) was added to the extract followed by concentrated sulfuric acid (1 mL). Steroids were detected by adding 1 mL of acetic anhydride to 0.25 g of a methanolic extract of sample followed by 1 mL H₂SO₄. A color change from violet to blue or green indicated the presence of steroids. The test for anthraquinones was performed by combining 0.5 g of extract boiled with 10 mL sulfuric acid and filtered; the filtrate was then shaken with 5 mL chloroform and the chloroform layer was transferred to another tube and 1 mL of ammonia was added and the color change was observed. Detection of terpenoids was carried out by adding 2 mL of chloroform to 0.5 g of extract and then carefully adding concentrated sulfuric acid (3 mL) to form a layer with a reddish to brown color at the interface.

High performance thin layer chromatography analysis

A finger print profile of the DAG extract was established using high performance thin layer chromatography analysis (HPTLC) (CAMAG, Switzerland). The method was described by Rajani, *et al.* The gum extract was dissolved in methanol (100 µL sample + 900 µL methanol) as stock solution. 10 µL of solution was applied on a precoated plate (Silica gel 60F 254) using a TLC spotter (CAMAG Automatic TLC Sampler 4 (ATS4)) and developed in a solvent system of n-hexane:ethylacetate:methanol (8:2:1 v/v). The plate was dried and analyzed with a TLC scanner (CAMAG Scanner 3) at 254 and 366 nm, and chromatograms and absorption spectra were recorded (9).

Chemicals

PTZ (Sigma, USA), diazepam (Darupakhsh, Iran) and flumazenil (Darupakhsh, Iran) were purchased from commercial sources.

Experimental studies

Anticonvulsant activity

Seizures were induced in mice with the standard convulsing agent, PTZ (60 mg/kg,

i.p.) according to the method described by Corda, *et al.* (16). The animals were randomly divided into five groups of 6 each. Group I served as the control and received normal saline (10 mL/kg, i.p.), Group II was treated with diazepam (1 mg/kg, i.p.), as a positive control group and groups III to V received DAG at doses of 500, 700, 1000 mg/kg i.p. After 30 min, all groups received PTZ. Animals which did not exhibit seizure after 30 min were considered as protected. The latency and duration of seizures were recorded 30 min after PTZ injection in the unprotected animals. Also, animals were observed for mortality for 24 h after PTZ administration.

The percentage protection was determined comparing the gum solution to diazepam, as established anti-seizure agent. The protection of diazepam considered to be 100 % and other agents were compared to diazepam.

Effect of flumazenil on the anticonvulsant activity of D. ammoniacum gum

The effect of flumazenil, a benzodiazepine receptor antagonist, on the anticonvulsant activity of DAG was studied in order to investigate the possible involvement of benzodiazepine receptors (17). Six groups of mice were selected (ten mice in each group); the first group, was given flumazenil (2 mg/kg) 5 min before the administration of DAG (700 mg/kg, i.p.) and 35 min before the injection of PTZ. In the second group, the animals received flumazenil (2 mg/kg) 5 min before the administration of diazepam (1 mg/kg). Also, three groups were injected with diazepam (1 mg/kg, i.p.), flumazenil (2 mg/kg) or normal saline 30 min before the administration of PTZ (60 mg/kg, i.p.) respectively. The latency and duration of convulsions at 30 min after injection of PTZ,

protection percentage (the percentage which is determined comparing to diazepam as established anti-seizure agent) and also mortality rate during the first 24 h were recorded. The anticonvulsant activity of DAG and diazepam in mice pretreated with flumazenil was assessed and compared with normal saline (10 mL/kg), flumazenil (2 mg/kg), diazepam (0.5 mg/kg) and DAG (700 mg/kg, i.p.) treated animals.

Effect of naloxone on the anticonvulsant activity of D. ammoniacum gum

Four groups of mice were selected (six mice in each) for further investigation of the possible involvement of opioid receptors in the anticonvulsant activity of DAG (18,19). Naloxone, an opioid receptor antagonist, was given at a dose of 5 mg/kg, 5 min before the administration of DAG (700 mg/kg i.p.) and 35 min before the injection of PTZ in groups of six mice each. The anticonvulsant activity of DAG in the groups pretreated with naloxone was assessed and compared with animals pretreated with DAG alone (700 mg/kg i.p.), with the naloxone (5 mg/kg) and normal saline (10 mL/kg) groups.

Statistical analysis

The results are reported as mean \pm S.E.M and tested with one way ANOVA followed by the multiple comparison test of Tukey-Kramer. Results with $P < 0.05$ were considered significant.

RESULTS

Preliminary phytochemical screening

The values obtained from the preliminary phytochemical study of DAG is described in Table 1.

Table 1. The results of a qualitative phytochemical screening of *D. ammoniacum* gum.

Compounds	Intensity
Triterpenes/steroids	+++
Alkaloids	-
Tannins	-
Flavonoids	++
Saponins	-
Anthraquinones	+
Coumarins	++
Reducing sugar	+

+++ , strong intensity reaction; ++, medium intensity reaction; +, weak intensity reaction; -, nondetectable

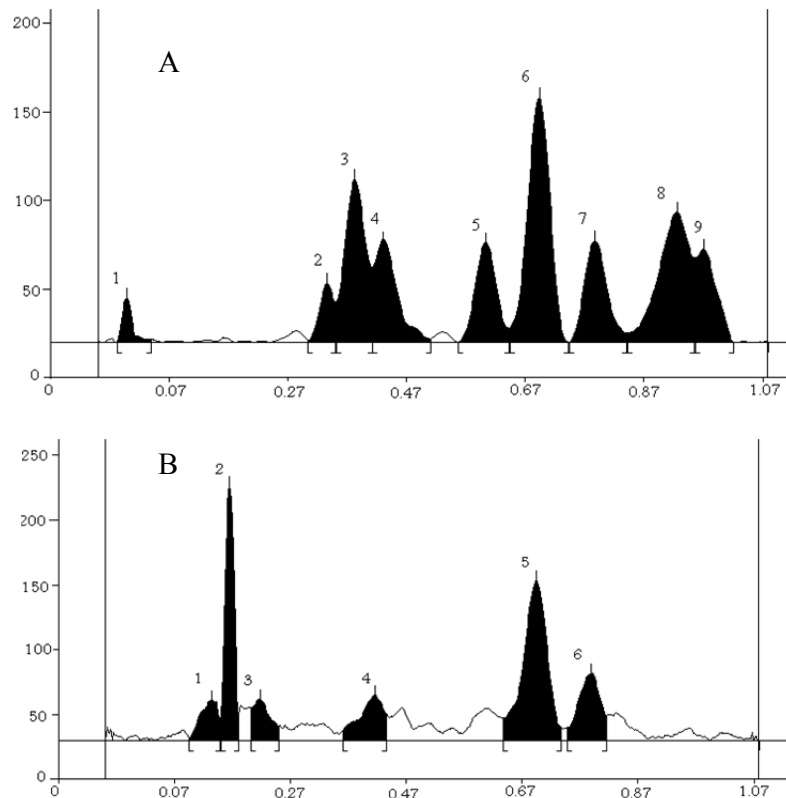


Fig. 1.High performance thin layer chromatography analysis of *D. ammiacum* gum extract. Thin layer chromatography analysis scanned at (A) UV 254 nm and (B) UV 366 nm.

The TLC fingerprint of D. ammiacum gum

Ammiacum gum extract was identified by TLC fingerprinting and the details are given in Table 2 and Fig. 1. The TLC fingerprint was comparable to that found in a previous study on DAG(9).

Anticonvulsant activity on pentylenetetrazole induced seizure

Injection of the DAG (500 mg/kg) significantly prolonged the onset of seizure activity ($P < 0.05$) and decreased seizure duration ($P < 0.01$) compared to the group treated with saline.

Furthermore, DAG at doses of 700 mg/kg and 1000 mg/kg also prolonged latency and decreased duration significantly ($P < 0.001$). In addition, DAG at doses of 700 and 1000 mg/kg, exhibited protection against seizures in 33% of animals and reduced mortality when compared to the control group.

Diazepam (1 mg/kg) protected 100% of the mice from PTZ-induced seizures and mortality (Table 3).

The effect of flumazenil on the anticonvulsant activity of D. ammiacum gum

Administration of flumazenil (2 mg/kg) 5 min before DAG (700 mg/kg) and 20 min before the injection of PTZ resulted in inhibition of the prolongation of latency and reduction in the duration of seizures exhibited by the gum solution ($P < 0.01$). Thus, flumazenil attenuated the action of DAG to some extent. Flumazenil also antagonized the anticonvulsant activity of diazepam (Table 4).

The effect of naloxone on the anticonvulsant activity of D. ammiacum gum

In the experimental group receiving naloxone (5 mg/kg) before DAG (700 mg/kg) and PTZ, there were no significant changes in latency or duration of seizures compared to the control group. Naloxone reduced the protective effects of DAG in prolonging seizure latency and reducing the duration of seizures and this inhibitory effect was more pronounced than that of flumazenil (Table 5).

Table 2.Thin layer chromatography fingerprint of *D. ammoniacum* gum.

Peak	Scanned at 254 nm*		Scanned at 366 nm	
	Rf	Relative (%)	Rf	Relative (%)
1	0.04	2.02	0.15	7.05
2	0.35	3.65	0.18	22.73
3	0.41	14.91	0.25	7.21
4	0.51	10.08	0.44	10.74
5	0.64	9.21	0.74	37.81
6	0.74	23.96	0.82	14.46
7	0.84	10.14		
8	0.96	17.87		
9	1.02	8.17		

*Fluorescent-quenched bands.

Table 3.Effect of *D. ammoniacum* gum solution on pentylenetetrazole-induced seizures in mice (data expressed as mean \pm S.E.M., n = 6).

Treatment group	%Protection (30 min)	Seizure latency (s)	Seizure duration (s)	Mortality (24 h)
PTZ + N.S (10 mL/kg)	0	78.17 \pm 11.21	40.3 \pm 2.60	3/6
PTZ + diazepam (1 mg/kg)	100	-	-	0/6
PTZ + DAG(500 mg/kg)	0	383.3 \pm 29.13*	26.0 \pm 1.50**	0/6
PTZ + DAG(700 mg/kg)	33	1508 \pm 103.0***	7.00 \pm 3.24***	0/6
PTZ + DAG(1000 mg/kg)	33	1521 \pm 90.20***	4.83 \pm 2.50***	0/6

N.S, normal saline; PTZ, pentylenetetrazole; DAG, *D. ammoniacum* gum aqueous solution. Percentage protection calculated in comparison to diazepam which has 100% seizure protection. *** P < 0.001 compared to the control group. ** P < 0.01 compared to the control group. * P < 0.05 compared to the control group.

Table 4.Effect of flumazenil on the anticonvulsant activity of *D. ammoniacum* gum solution in pentylenetetrazole-induced convulsion in mice (data expressed as mean \pm S.E.M., n = 6).

Treatment group	%Protection (30 min)	Seizure latency	Seizure duration (s)	Mortality (24 h)
PTZ + N.S (10 mL/kg)	0	78.17 \pm 11.2	40.33 \pm 2.6	3/6
Flumazenil (2 mg/kg)	0	75.33 \pm 6.11	31.67 \pm 2.3	2/6
Diazepam (1 mg/kg)	100	-	-	0/6
Diazepam + Flumazenil	0	122.0 \pm 10.4	33.83 \pm 3.5	2/6
DAG(700 mg/kg)	33	1508 \pm 103***	7.00 \pm 3.2***	0/6
DAG(700 mg/kg) + Flumazenil	33	403.7 \pm 99.2**,#	25.50 \pm 2.8**,#	1/6

N.S, normal saline; PTZ, pentylenetetrazole; DAG, *D. ammoniacum* gum aqueous solution. Percentage protection calculated in comparison to diazepam which has 100% seizure protection.** P < 0.01 compared to the control group. *** P < 0.001 compared to the control group. # P < 0.001 compared to the DAG (700 mg/kg) group.

Table 5.Effect of naloxone on the anticonvulsant activity of *D. ammoniacum* gum solution in pentylenetetrazole induced convulsion in mice (the data were expressed as mean \pm S.E.M., n = 6).

Treatment group	%Protection (30 min)	Seizure latency (s)	Seizure duration (s)	Mortality (24 h)
PTZ + N.S (10 mL/kg)	0	78.17 \pm 11.2	40.33 \pm 2.60	3/6
Naloxone (5 mg/kg)	0	48.67 \pm 3.1	56.17 \pm 3.64	4/6
DAG (700 mg/kg)	33	1508 \pm 103 [§]	7.00 \pm 3.24 [§]	0/6
DAG + Naloxone	0	134.8 \pm 2.6 [#]	48.83 \pm 4.10 [#]	2/6

N.S, normal saline; DAG, *D. ammoniacum* gum aqueous solution. Percentage protection calculated in comparison to diazepam which has 100% seizure protection. [§] P < 0.001 compared to the control group. [#] P < 0.001 compared to the DAG (700 mg/kg) group.

DISCUSSION

Given that the treatment of epilepsy with currently available antiepileptic medications is frequently sub-optimal, interest in discovering

new alternative drugs from natural sources has increased (5,20). There are some herbs and herbal compounds which showed anticonvulsant activity (16). Iranian traditional medicine has utilized some herbs or herbal preparations such

as *D. ammoniacum* (Called Oshagh) for the treatment of epilepsy (7,9).

Ammoniacum gum has been traditionally used for different purposes in Iran. It is also used for some CNS disorders such as epilepsy and for reducing joint pain (7,12,21). Previous studies showed antimicrobial, cytotoxic and acetylcholinesterase inhibitory activity of *Dorema ammoniacum* (22-24)

The TLC fingerprinting of ammoniacum gum was confirmed using HPTLC and it was comparable to that from a previous study on ammoniacum gum (9). In this study, DAG produced a statistically significant reduction in seizures' duration and an increase in the latency period of seizures induced by PTZ in the mouse model. This effect was appeared in all doses used. No death was recorded even at highest dose of DAG used (1000 mg/kg). This suggested that the gum aqueous solution is relatively safe and non-toxic in mice even at the highest dose used in this study.

The main mechanism of PTZ-induced seizures involves the reduction in the level of γ -aminobutyric acid (GABA) in the cortex. GABA is considered to be a major inhibitory neurotransmitter in the CNS of mammals and decreased GABA activity has been implicated in convulsions, as GABA mediates the inhibition of neuronal responsiveness and activity by increasing chloride-ion conductance through the opening of the chloride-ion channel (16,25). The findings of the present study suggest that DAG might have delayed the occurrence of PTZ-induced seizure activity and reduced its duration by acting on the GABAergic system. Since opiates have been involved in many functions in CNS, naloxone was used to evaluate the possible role of opioid system in effects of DAG. Naloxone decreased the prolongation of seizure latency induced by DAG and also antagonized the effect of DAG on decreasing the duration of seizures in the PTZ model. This attenuation of the anticonvulsant effect of DAG by an opiate receptor antagonist suggests the involvement of opiate receptor activation in the effects of DAG (26-28). Administration of flumazenil before DAG led to an increase in the latency and a reduction in the duration of seizures compared to the control group. This shows that

flumazenil antagonizes the effect of DAG on latency time and duration of seizures in the PTZ model. These results, suggest that the anticonvulsant effect of DAG probably involves activation of benzodiazepine receptors. Since benzodiazepine receptors are present on the GABA receptor complex, it can be deduced that the effect of DAG might involve binding sites modulating the activity of GABA receptor complex. It has been found that many flavonoids can act as benzodiazepine-like molecules in the central nervous system (CNS) and modulate GABA-generated chloride currents in animal models of anxiety, sedation and convulsion (29). It is possible that the anticonvulsant activities of DAG are related to its flavonoid content (Table 1). Finally the results obtained from this work suggested that an aqueous solution of DAG had anticonvulsant activity, hence supports the traditional use of the plant in the treatment of convulsive disorders. This observed activity of the plant extract on PTZ-induced seizures could be due to the involvement of the GABAergic pathway. Further investigation is required to determine which components in the DAG extract may be responsible for the observed activity and determine their specific mechanism of action (25).

CONCLUSION

This study showed that DAG had significant anticonvulsant activity in PTZ-induced seizures, in a dose dependent manner via involvement of GABAergic and opioid systems. More studies are needed to further elucidate its detailed underlying mechanism.

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