Antiparasitic Effects of Plant Species from the Diet of Great Bustards

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Research

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Abstract

Background: Diets combine food types according to some trade-offs, as for example maximising nutrients and minimising toxins. But some diets include elements because of their activity against the host parasites and other pathogens. This so-called medicinal role of food is under-reported in the literature, either because toxic elements in diets of livestock and wildlife are infrequent, or because their activity against parasites and pathogens has not been fully documented. We contribute to fill this knowledge gap by testing the activity of extracts and essential oils from *Papaver rhoeas* and *Echium plantagineum* against a selection of laboratory pathogens. These plants are strongly selected by great bustards *Otis tarda* during the mating season.

Results: During this season we found a significantly higher frequency of *P. rhoeas* in male than in female faeces. The activity of different extracts of these plants against some laboratory models including a flagellated protozoan (*Trichomonas gallinae*), a nematode (*Meloidogyne javanica*) and a fungus (*Aspergillus niger*) was evaluated. We found activity against nematodes and trichomonads in non-polar and polar extracts of the aerial parts of *P. rhoeas,* especially the extracts of flowers and capsules, and *E. plantagineum*, especially the extracts of leaves and flowers.

Conclusions: Both plants showed anti-parasitic activity, a result compatible with the hypothesis that great bustards eat plants for non-nutritional purposes, likely to assist them in coping with parasites and other pathogens, and *P. rhoeas* could be especially helpful for males during the mating season, when their immune system is weakened by the investment in secondary sexual characters and sexual display. The self-medication properties of plants and animals included in diets should be considered in studies of foraging behaviour, habitat selection, and even conservation biology of wildlife.

Background

Several studies have reported anti-parasitic effects of plants both in livestock [1 and references therein, 2] and wild animals [3, 4]. These effects are usually due to secondary metabolites such as terpenes, alkaloids, glycosides and tannins [5], which are typically non-nutritional and frequently have toxic or even poisonous properties [6]. To distinguish whether these substances are ingested inadvertently or as a means to confront infectious diseases would require very detailed behavioral observations that are extremely difficult to record in wild species under usual field conditions [7]. However, obtaining evidences of their antiparasitic effects is a precondition to consider them involved in a self-medication process.

One of the species in which self-medication has been suggested is the Great Bustard (*Otis tarda*). García-Montijano et al. [8], reported that parasitic and fungal infections could cause mortality of free-living Great Bustards in Spain (14.3% of death in juveniles was due to small intestine obstruction with cestodes, and fungal pneumonia and air saculitis by *Aspergillus fumigatus* were responsible for 16.7% of death in adults). Bustards are affected by a wide range of pathogens and parasites, including numerous bacteria, protozoa, helminths and arthropods. The protozoans *Eimeria* spp., *Cryptosporidium* spp., *Giardia* spp., *Trichomonas* spp., *Histomonas* spp. and *Hexamita* spp. are commonly present in their digestive tracts [9,

10]. *Trichomonas gallinae* and *Entamoeba anatis* cause oropharyngeal diseases in bustards [11]. Cestodes (*Hispaniolepis* sp., *Raillietina cesticillus*, *Schistometra* (*Otiditaenia*) *conoides*, and *Idiogenes otidis*), nematodes (*Capillaria* sp., *Syngamus trachea*, *Cyathostoma* sp., *Heterakis gallinae*, *H. isolonche Aprocta orbitalis*, *Oxyspirura hispanica*, and *Trichostrongylus* sp.), insects (including mallophagan such as *Otilipeurus turmalis*, and fly maggots such as *Lucilia sericata*) and ticks (*Rhipicephalus sanguineus*, and *Hyalomma* sp.) also infest bustards [9, 10, 12, 13].

In previous studies [9, 14] we investigated the antiparasitic and antimicrobial effects of blister beetles (Meloidae), which are avoided by most animals but consumed by great bustards. Blister beetles contain cantharidin, a highly toxic monoterpene that could have positive effects in controlling the parasite load of the host [9]. We found that great bustards males consumed more blister beetles than females, which suggested that males could use cantharidin to reduce their parasite load and increase their sexual attractiveness [14].

Although great bustards eat beetles and other invertebrates in spring and summer, their diet is mostly vegetarian over the whole year, including mainly leaves of green plants [15–17]. Some of these plants could also have properties that might help great bustards control their parasite load. For example, Lane et al. (1999) found that most weeds were consumed in proportions expected by their abundance, but noted two exceptions, the common poppy *Papaver rhoeas* and the purple viper's bugloss *Echium plantagineum*. The diet of great bustards showed much higher foraging ratios [18] of these two plants in April compared to other months (Fig. 1). Since April is the month when males reach the peak of display activity and most matings occur [19, 20], we hypothesize that consumption of these two plants could help them reduce the negative effects of parasites during this important phase of their reproductive cycle, as is the case with blister beetles. Males could benefit from the medicinal properties of these plants during a period when they are subjected to high stress and reduced immune resistance to infections due to their strenuous investment in sexual display [19–22]. Parasite over-loads may indeed affect the fitness or reproductive status of the host [23, 24], and chemical compounds helping to control parasites when the threat of parasitism is greater may have benefits on health and reproductive success [25, 26].

To test the hypothesis, our present study had the following objectives. First, we examined whether consumption of *P. rhoeas* and *E. plantagineum* is male-biased. A higher proportion of these plants in the diet of males would support the hypothesis that males use them to reduce their parasite load during their strenuous display season, in a similar way as that suggested for cantharidin [14]. Alternatively, or complementary to a self-medication function affecting only males, both plants might also have a prophylactic function for females during the peak copulation period in April when exposure to sexually transmitted diseases is highes. Second, we investigated whether extracts and essential oils (EOs) from *P. rhoeas* and *E. plantagineum* have antiparasitic activity against the flagellated protozoa *Trichomonas gallinae*, the endoparasitic nematode *Meloidogyne javanica*, and the fungus *Aspergillus niger*. Third, we analysed the chemical composition of extracts to identify what components could be active against pathogens. The fulfilment of these three objectives is not a demonstration of self-medication in Great bustards, but a necessary step towards it. A step that contributes to expand the frontiers of zoology.

Results

Plant consumption

During the mating season the dry weight proportion of *Papaver rhoeas* was higher in male faeces (24.9 ± 22.8%) than in females faeces (21.5 ± 23.6%) (Table 1 and [17]). The sex difference was not significant in the non-mating season (males: 7.3 ± 13.7%, females: 6.7 ± 15.8%, Table 1 and [17]). As for *Echium plantagineum*, the dry-weight proportion in male faeces (0.9 ± 0.2%) was marginally ($p < 0.10$) higher than in female faeces (0.1 ± 0.5%) in the mating season, and higher in the non-mating season (males: 1.5 ± 4.4%, females: 0.6 ± 3.8, Table 1 and [17]).

Table 1
GLMM results of sex difference in mating and non-mating seasons of great bustards

Plant	Season	χ^2	df	p	Estimate ± SE
P. rhoeas	mating	50.2	1	< 0.01	sex (male): 0.29 ± 0.04
					intercept: -2.09 ± 0.77
	non mating	0.1	1	0.80	sex (male): 0.01 ± 0.04
					intercept: -3.27 ± 0.66
E. plantagineum	mating	3.3	1	0.07	sex (male): 0.77 ± 0.43
					intercept: -8.29 ± 0.76
	non mating	67.9	1	< 0.01	sex (male): 0.93 ± 0.11
					intercept: -6.44 ± 0.77

Bioactivity of plant extracts

Using different solvents, a total of 17 extracts were obtained (Table 2) containing low polarity (hexane, Hex), medium polarity (ethyl acetate, EtOAc), polar (ethanol, EtOH and methanol, MeOH,, dichloromethane infusion IDCM), water soluble (infusion, I, infusion-freeze dried, IFD) and volatile (essential oil, EO) compounds from the plants. The highest yields corresponded to the methanolic / ethanolic extracts. *P. rhoeas* flowers (MeOH and I) gave higher yields than *E. plantagineum* flowers.

Table 2
Extracts and yields from *P. rhoeas* and *E. plantagineum*

Plant part	Extract	Extraction method	Yield (%)	
			P. rhoeas	E. plantagineum
Leaves	Hexane (Hex)	Soxhlet (solid-liquid)	2.38	2.41
	Ethyl acetate (EtOAc)	Soxhlet (solid-liquid)	1.94	3.15
	Ethanol (EtOH)	Soxhlet (solid-liquid)	10.65	7.13
Flowers	Methanol (MeOH)	Soxhlet (solid-liquid)	20.81	6.86
	Essential oil (EO)	Clevenger distillation	0.02	0.01
	Infusion-freeze dried (IFD)	Clevenger / freeze-dried	2.70	0.80
	Infusion-DCM (IDCM)	Clevenger / liquid-liquid (DCM)	0.06	0.05
Capsules	Hexane (Hex)	Soxhlet (solid-liquid)	5.92	-
	Ethyl acetate (EtOAc)	Soxhlet (solid-liquid)	3.71	
	Ethanol (EtOH)	Soxhlet (solid-liquid)	11.46	

The extracts were tested *in vitro* against a range of parasites. Table 3 shows their effects on *T. gallinae* and *A. niger* (mycelial growth). *T. gallinae* was the most sensitive target organism, with *P. rhoeas* being more active than *E. plantagineum*. Among different *P. rhoeas* extracts, flowers-EO (F-EO), flowers-IFD (F-IFD)and capsules-Hex (C-Hex) showed strong trichomonacidal effects, followed by flowers-MeOH (F-MeOH) and leaf-EtOH (L-EtOH) extracts (Table 3). The most active extracts from *E. plantagineum* were leaf-EtOH (L-EtOH) and F-IFD, followed by F-EO and leaf-EtOAc (L-EtOAc). Therefore, the trichomonacidal compounds could be (1) volatiles and apolar compounds present in the flowers and capsules of *P. rhoeas* (EO / Hex), polar compounds present in the flower infusion-freeze dried (IFD) of *P. rhoeas* and *E. plantagineum* and non-volatile polar compounds present in the L-EtOH leaf extract of *E. plantagineum*.

Table 3
Activity of *P. rhoeas* and *E. plantagineum* extracts on *T. gallinae* and *A. niger*

Plant part	Extract	*T. gallinae*			*A. niger* (1 mg/ml)	
		µg/ml	Pr	Ep	Pr	Ep
Leaves	Hex	400	33.4 ± 3.8	28.8 ± 3.6	3.3 ± 2.7	0.0 ± 5.4
	EtOAc	400	51.8 ± 6.8	56.7 ± 7.2	0.0 ± 7.4	10.0 ± 6.4
	EtOH	400	71.1 ± 6.2	95.0 ± 11.5	6.0 ± 3.9	5.8 ± 5.8
		200	34.8 ± 3.1	34.8 ± 3.3		
		100	26.5 ± 4.3	17.2 ± 1.3		
Flowers	EO	400	98.5 ± 13.3	67.3 ± 12.4	9.6 ± 0.5	2.4 ± 0.0
		200	96.4 ± 13.3	37.7 ± 6.9		
		100	65.8 ± 8.6	9.8 ± 1.8		
	MeOH	400	82.7 ± 4.8	38.4 ± 1.4	4.6 ± 0.3	29.6 ± 0.0
		200	37.1 ± 5.7			
		100	10.4 ± 0.4			
	IFD	400	98.6 ± 18.4	94.6 ± 23.5	24.1 ± 8.9	51.9 ± 8.6
		200	52.9 ± 10.4	46.6 ± 4.5		
		100	54.3 ± 2.6	25.9 ± 2.8		
	IDCM	400	30.2 ± 5.6	20.8 ± 4.0	17.8 ± 8.3	0.0 ± 3.5
Capsules	Hex	400	83.2 ± 4.3		11.6 ± 1.8	
		200	85.6 ± 9.7			
		100	56.8 ± 5.0			
	EtOAc	400	22.1 ± 3.8		2.1 ± 2.5	
	EtOH	400	48.9 ± 2.7		1.5 ± 0.6	

A. niger mycelial growth was moderately inhibited by the flower freeze dried infusion of *E. plantagineum* (IFD, 52%; Table 3). However, these extracts did not affect *A. niger* spore germination (data not shown).

The extracts were also tested on an endoparasite nematode model (*M. javanica*). The results are shown in Table 4. Freeze dried flower infusions from both plant species demonstrated strong nematicidal effects (up to 5% dilutions). Among organic extracts, the MeOH flower extract of *E. plantagineum* was also active against *M. javanica* (81.3%).

Table 4
Activity of *P. rhoeas* and *E. plantagineum* on *M. javanica*

Plant part	Extract/EO	mg/ml	Pr	Ep
Leaves	Hex	1	0.2 ± 0.6	3.3 ± 1.2
	EtOAc	1	3.9 ± 1.7	3.3 ± 1.1
	EtOH	1	18.4 ± 2.5	4.0 ± 1.0
Flowers	EO	1	2.3 ± 0.3	5.2 ± 0.9
	MeOH	1	19.8 ± 5.9	81.3 ± 2.8
		0.5		14.0 ± 4.0
	IFD [a]	100%	100.0	100.0
		50%	100.0	100.0
		25%	21.0 ± 2.0	1.7 ± 1.7
	IDCM	1	10.7 ± 1.7	20.4 ± 5.2
Capsules	Hex	1	16.7 ± 3.2	
	EtOAc	1	15.5 ± 2.0	
	EtOH	1	0.0 ± 0.3	

Table 5

A. LC-MS analysis of *P. rhoeas* polar extracts

Retention time (min)	Base Peak m/z	$[M]^+$ Adduct form	% Area				Category / alkaloid tentative identification
			Leaf EtOH	Flower MeOH	Flower IFD	Flower IDCM	
1–10			69.52	67.95	92.15	0.00	Polar compounds
10–50			20.61	17.88	5.67	33.07	Medium polarity compounds
			9.87	14.18	2.18	66.93	Identified alkaloids
17.73	339 317	316 $[M + Na]^+$	3.97				(S)-3'-Hydroxy-Nmethylcoclaurine
22.52	370 392	369 $[M + H]^+$			1.64	36.17	Rheagenine / Cryptopine
25.92	370 392	369 $[M + H]^+$				12.71	Rheagenine / Cryptopine
26.76	338	337 $[M + H]^+$	1.89	0.69		1.93	Dihydroberberine
27.06	386 368	385 $[M + H]^+$				6.75	Glaucamine
28.32	400 422	399 $[M + H]^+$				1.74	Glaudine
28.70	384 406	383 $[M + H]^+$	2.47	7.90		0.89	Rhoeadine /isorhoeadine
30.23	370 392	369 $[M + H]^+$	1.54	2.63	0.54	6.74	Allocryptopine
33.65	384	383 $[M + H]^+$		2.96			Cowlteropine

In short, *P. rhoeas* contains antiprotozoal and anthelmintic compounds, while *E. plantagineum* contains components with antiprotozoal, anthelmintic and, to a lesser extent, antifungal activity.

Chemical analysis

Volatile (EO) and non-polar (Hexane) extracts:

The GC-MS analysis of the essential oil (EO) from *P. rhoeas* flowers showed the presence of alkanes including n-hexatriacontane and related (36.3%), lipids such as palmitic acid (11.13%), 1-eicosanol and

related (11.13%), methyl palmitate (3.39%), ethyl palmitate (2.98%), di-(9-octadecenoyl)-glycerol (2.80%), ethyl linoleate (1.61%), 2-(9,12-octadecadienyloxy)-ethanol (1.40%) and myristic acid (1.40%).

The flower capsules of *P. rhoeas* also contained lipid-related compounds in the Hex extract, such as methyl linoleate (39.12%) / ethyl linoleate (3.36%), 1-eicosanol (17.39%), palmitic acid (7%) and the sterol stigmast-5-en-3-ol (4.49%). Their EtOAc extract contained 2-monopalmitin (24.67%), 2-monostearin (13.52%), stigmast-5-en-3-ol (11.43%) and other sterols (9.25%), myristic acid (6.11%), neophytadiene (5.94%), 2-chloroethyl linoleate (3.85%) and myristaldehyde (2.06%).

The EO from *E. plantagineum* flowers showed a high content in alkanes including n-hexatriacontane and related (76.27%), 2-monopalmitin (11.13%), docosane (6.43%), hexadecanoic acid (2.98%), dimethyl ester of docosan-1,22-dioic acid (2.35%), 6,10,14-trimethyl-2-pentadecanone (2.11%), tritetracontane (1.18%).

Polar extracts (EtOH / MeOH, infusion):

The HPLC-MS analysis of the polar extracts (leaves ethanolic, L-EtOH; flowers methanolic F-EtOH; flower infusion lyophilized, F-IFD; dichloromethane extract of flower infusion, F-IDCM) are shown in Tables 5A (*P. rhoeas*) and 5B (*E. plantagineum*). To categorize the extracts, the peaks have been arbitrarily grouped as polar and medium polarity compounds according to their retention times (1–10 min and 10–50 min). The alkaloidal components identified in these extracts eluted between 10–50 min as part of the medium polarity class.

Among *P. rhoeas* extracts, the freeze-dried infusion of flowers contained a high percentage of polar components (92%). The alcoholic extracts had similar amounts of polar (L-EtOH and F-MeOH, 69 – 68%) and medium polarity peaks (21 – 18%), with more alkaloids identified in the flowers (14%). However, most of the identified alkaloids were concentrated in the F-IFD partitioned with dichloromethane (F-IDCM, 67%, see Table 5A).

These extracts showed the presence of molecular ions compatible with previously reported alkaloids [27]. The leaf EtOH extract showed (S)-3'-hydroxy-N-methylcoclaurine-like ions (4%), followed by rhoeadine /isorhoeadine (2.5%), dihydroberberine (2%), and allocryptopine (1.54%). The flower MeOH extract showed rhoeadine /isorhoeadine (8%), allocryptopine and cowlteropine (3%) compatible ions. The IDCM of the flower infusion concentrated most of the alkaloid-like compounds, showing molecular ions compatible with rheagenine / cryptopine (49%), glaucamine / allocryptopine (7%), dihydroberberine (2%), rhoeadine /isorhoeadine (0.9%) and dihydroberberine (0.7%). The freeze-dried flower infusion IFD showed molecular ions compatible with rheagenine / cryptopine (1.64%) and allocryptopine (0.54%, see Table 5A).

E. plantagineum alcoholic extracts (L-EtOH / F-MeOH) contained different amounts of polar components (73 / 54%) and similar amounts with medium polarity (19 / 18%). Most of the identified alkaloids were concentrated in the flower organic extracts (F-MeOH / IDCM, 31 / 40%, see Table 5B).

Several molecular ions compatible with pyrrolicidine alkaloids (PAs) reported in *E. plantagineum* [28–31] were identified in these extracts. Overall, echimidine-like molecular ions were the most abundant. The leaf EtOH extract contained echimidine (5.8%) and echiumine-Nox (1.87%) molecular ions. The flower MeOH showed molecular ions for echimidine (13,7%), intermedine (5,2%), heliotrine (4.7%), lycopsamine (3%), heliotrine-Nox (2.6%), and echiumine-Nox (1,5%). The flower IDCM extract showed molecular ions compatible with echimidine (25.6%), echiuplatine (5,07%), echimidine-Nox (4.15%), acetyllycopsamine (3,1%), echiumine-Nox (1,24%) and intermedine (1,12%). The flower IFD extract showed molecular ions compatible with echimidine (4.93%), intermedine (2.62%), heliotrine-Nox (1.72%), and echiumine-Nox (1,18%, see Table 5B).

Table 5

B. LC-MS analysis of E. plantagineum extracts

Retention time (min)	Base Peak m/z	$[M]^+$ Adduct form	% Area				Category / alkaloid tentative identification
			Leaf EtOH	Flower MeOH	FlowerIFD	Flower IDCM	
1–10			72.69	54.14	76.51	0.00	Polar compounds
10–50			19.60	18.16	13.02	66.97	Medium polarity compounds
			7.71	30.72	10.45	40.27	Identified alkaloids
7.58	330 352 168	329 $[M + H]^+$		2.63	1.72		Heliotrine NOx
17.40	314 336	313 $[M + H]^+$		4.67			Heliotrine
18.48	300 322	299 $[M + H]^+$		5.23	2.62	1.12	Intermedine
19.33	300 322	299 $[M + H]^+$		3.01			Lycopsamine
23.43	390 342 364	341 $[M + H]^+$				3.09	Acetyllycopsamine
26.97	398 420	397 $[M + H]^+$	5.84	13.71	4.93	25.60	Echimidine
27.17	414 436	413 $[M + H]^+$				4.15	Echimidine NOx
30.49	382 404	381 $[M + H]^+$				5.07	Echiuplatine
30.87	398 420	397 $[M + H]^+$	1.87	1.47	1.18	1.24	Echiumine-NOx

Discussion

We found antiparasitic effects in *Papaver rhoeas* and *Echium plantagineum*, two plants strongly selected by great bustards during the mating season. From these plants we obtained two categories of active extracts: apolar (EO and Hex) and polar (alcoholic: EtOH / MeOH and aqueous infusion: IFD). We determined their chemical composition and tested their activity on a sample of common laboratory pathogens. Based on these results and a review of the antipathogenic properties of both plants, we infer that great bustards feed on them to reduce their load of pathogens.

Papaver rhoeas

Non-polar and polar extracts of aerial parts of *P. rhoeas*, especially flowers and capsules, showed activity against nematodes and trichomonads. Among non-polar extracts, the flower essential oil (EO) and the capsule hexane extract showed strong trichomonacidal effects, the effect of EO being particularly powerful. A study of *P. rhoeas* EO from aerial parts collected in Turkey showed phytol (52.8%), tricosane (7.8%), 2-pentadecanone (6%) and heneicosane (5.3%) as the major compounds [32], while the EO studied here was characterized by alkanes such as n-hexatriacontane and 1-eicosanol and the fatty acid palmitic acid. The non-polar extract (Hex) of the capsules mostly contained methyl / ethyl linoleate, 1-eicosanol and palmitic acid. Palmitic acid has been reported as being the main component of non-polar (Hex) extracts of *P. rhoeas* leaves [33].

Previous results have shown trichomonacidal effects on *T. vaginalis* of *Nigella sativa* seed oil containing fatty acids [34]. However, this is the first report on trichomonacidal effects of *P. rhoeas* lipid extracts. Trichomonads are unable to biosynthesize fatty acids and cholesterol but can incorporate these compounds from the medium without further modification [35]. Therefore, externally supplied fatty acids and sterols could interfere with Trichomonads lipid metabolism.

The bioactivity of *P. rhoeas* polar extracts correlated with their content in polar compounds (peaks with retention times of < 10 min). The alcoholic ones (L-EtOH, F-MeOH) showed trichomonacidal effects, while the F-IFD showed potent trichomonacidal and nematicidal effects. Nematicidal activity has been previously reported for aqueous extracts (4%) of *P. roheas* leaves against *M. javanica* [36]. These extracts contained molecular ions compatible with reported *Papaver* alkaloids [27], mostly concentrated in the dichloromethane partition of the flower infusion (F-IDCM), with rheagenine / criptopine, rhoeadine and hydroxy-N-methyl-coclaurine ions being the most abundant in the F-IDCM, F-MeOH and L-EtOH extracts. Since the alkaloid-rich F-IDCM partition was not active, the trichomonacidal /nematicidal effects of *P. rhoeas* polar extracts cannot be attributed to these alkaloids, indicating that more polar components of the extracts are responsible for the observed effects. Among *P. rhoeas* components, alkaloids are the most representative, especially (+)-rhoeadine, along with N-methylasimilobine, rhoeagenine, epiberberine and canadine, depending on the plant origin [37]. Minor alkaloids included roemerine [37], with reported antibacterial, antifungal and anthelmintic activities [38, 39]. Furthermore, alkaloids such as allocryptopine, potopine and berberine were nematicidal against *Strongyloides stercolaris* larvae [40]. *P.*

rhoeas also contains flavonoids, phenols, organic acids and vitamin C [41–43]. Flavonoids may reduce the oxidative stress and enhance immunity, so they are selected by different bird species, presumably as a prophylactic drug [44] against pathogens. Polyphenols regulate immune and inflammatory responses during enteric bacterial and parasitic infections in livestock [45], and organic acids can significantly reduce microbial contamination in turkeys [46].

Corn poppy has been used since ancient times as a food ingredient and traditional remedy [37], but cases of poisoning with *P. rhoeas* in adults, children and animals have been described [47, 48]. Poppy poisoning in humans can cause nausea, vomiting, altered mental state, headache, convulsions, miotic pupils, lethargy and disorientation [49]. *Papaver* species are actively toxic or narcotic and unpalatable to grazing animals. Animals are safe since the odour and taste of the plants render them obnoxious but there are reports of cattle and horses being poisoned by *P. rhoeas* [47]. Nonetheless, great bustards include *Papaver rhoeas* in their diet throughout most of the year and, to our knowledge, they are not poisoned by corn poppies. The diet composition of great bustards and the activity of *P. rhoeas* extracts shown here support the hypothesis of a self-medication function for this plant species during the bird's mating season [14].

Echium plantagineum

Extracts of the aerial parts of *E. plantagineum* (non-polar and polar) also showed trichomonacidal and nematicidal effects. The flower essential oil (EO) of *E. plantagineum*, with moderate trichomonacidal effects, was characterized by alkanes such as n-hexatriacontane and related substances, and the fatty acid ester 2-monopalmitin.

Similarly to *P. rhoeas* extracts, the bioactivity of *E. plantagineum* polar extracts also correlated with their content in high polarity compounds. The alcoholic F-MeOH showed trichomonacidal effects, while the freeze-dried infusion (IFD) showed potent trichomonacidal and nematicidal effects. Several molecular ions compatible with Pas reported in *E. plantagineum* [28–31] were identified in the polar extracts, mostly concentrated in the organic fraction of the flowers' infusion (IDCM). Echimidine molecular ions were the most abundant in all extracts. Since the PA-rich IDCM partition was not active, the trichomonacidal /nematicidal effects of *E. plantagineum* polar extracts cannot be attributed to these alkaloids. However, nematicidal effects of PAs on *Meloidogyne incognita* have been reported for heliotrine, lasiocarpine and monocrotaline, but these effects were dependent on the PA structure and the exposure period (168 h)[50].

E. plantagineum produces different classes of secondary metabolites, including pyrrolizidine alkaloids (PA) in the aerial parts and seeds [29, 51, 52], echimidine and echiumine N-oxide being particularly abundant [30]. Pas are easily reduced to free bases and are metabolized by the herbivorous cytochrome P-450 oxidases, which give rise to pyrrol alkylating intermediates. Reactive pyrroles damage cellular DNA and are dangerous to cattle, horses, sheep, pigs, and rats, affecting also humans [53–56]. Harmful effects on bird health have also been described as a result of PA consumption [57, 58].

Diet and health of great bustards

In this study we suggest that selection of *P. rhoeas* and *E. plantagineum* by great bustards could be based on the antipathogenic effects of these plants. The use of plants with active secondary metabolites for preventing or reducing parasite and pathogen loads (self-medication) has been described in invertebrates [59–61], mammals [62, 63] and birds [64–66]. As for great bustards, Bravo et al. [14] described for the first time a probable case of self-medication by ingestion of toxic insects. They found that bustards included two blister beetles of the family Meloidae in their diet. These beetles contain cantharidin, a highly toxic compound that can be even lethal for bustards if ingested in high doses [67]. Bravo et al. [14] found a male-biased consumption of blíster beetles, and interpreted it as a way to enhance their attractiveness to females by reducing their parasite load. Before selecting a mate for copulation, a female bustard carefully examines the cloaca of the displaying male and usually pecks it, probably looking for parasites. Bravo et al. [14] suggested that a higher consumption of blister beetles by males could be a sexually-selected mechanism to enhance their mating success. The hypothesis of self-medication in bustards was supported by Withman et al. [9], who demonstrated antiparasitic effects of extracts from blister beetles (*Berberomeloe majalis*) against different models (protozoan, nematodes, ticks and insects). Heneberg [68] proposed that bustard males self-medicated seeking sexual arousal rather than antipathogenic effects.

Regardless of the ultimate function of blister beetle selection, here we propose that there are more species with similar properties in the diet of great bustardst, and we present the antiparasitic results for two plant species as an example. Although the toxicity of *P. rhoeas* and *E. plantagineum* differs, great bustards show a marked selection for both plants during the mating season (Fig. 1), and during that season we found higher amounts of *P. rhoeas* in male than in female fecal samples (Table 1). Why could males be more interested in this plant than females and why during the mating season? Courtship is strenuous for males in most polygynous species and particularly in great bustard males, who show the most strongly skewed mating success reported among lekking birds, suggesting an extreme intensity of sexual selection in this species [20]. Males develop costly ornaments every spring and perform exhausting displays to attract females [19, 20]. It is known that physiological investment in sexually selected characters competes with investment in immune response [69], a trade-off which is not as demanding for females, with the consequence of smaller loads of parasites and pathogens in this sex compared to males [70]. Great bustard males would hold higher load of parasites and other pathogens than females and still sire a number of descendants in the next generation if females chose to mate them, according to the Handicap Principle [71, 72]. Attracting females while keeping pathogens may be quite demanding, so polar components in *P. rhoeas*' capsules and flowers would help males control pathogens, reduce fatigue [sensu 68], or both. Measuring these effects *in vivo* is beyond any feasible experimental setup, at least with current designs and legal restrictions on experimenting with this vulnerable species. But inferring the causal links is reasonable, so we put forward the challenging hypothesis that great bustard males disproportionately foraged on *P. rhoeas* during the mating season due to the effects of some non-nutritional compounds present in this plant.

Activity of *E. plantagineum* against pathogens tested in the present study was noticeably higher than that of *P. rhoeas*, but the proportion of *Echium plantagineum* dry weight in faeces of great bustards was about 50 times smaller than that of *P. rhoeas* during the mating season, and seven times smaller in the non-mating season. The harmful effects of pyrrolizidine alkaloids on bird health described in previous studies [57, 58] could explain the small amount of *E. palntagineum* in great bustard faeces, but not its higher proportional dry weight in males compared to females during the non-mating season, unless we admit that males would have a greater need of these compounds than females also outside the mating season. We cannot discard that males would also benefit more than females from the properties of *E. plantagineum* during the months we define as *non-mating* season (November-January, and July, see [17]). Males indeed start displaying and fighting to establish their dominance hierarchy in the male group in December-January [73], so these winter months could also be highly energy demanding. As for July, this is the hottest month of the year, when males suffer the debilitating effects of a much lower heat resistance compared to females [74], coinciding with the moult of the flight feathers.

Conclusions

In sum, we have shown that two plants consumed by great bustards are ingested in higher proportions by males, probably to cope with their higher stress levels due to their demanding display effort, and that both plants show activity against pathogen models in laboratory conditions. These results support to some extent the self-medication hypothesis [75] in these birds. Nonetheless, differentiating between a possible indirect effect of the ingestion of species rich in secondary metabolites or the consumption for their nutritional value is one of the difficulties in the interpretation of self-medication in animals [76]. For example, alkaloids and other phytochemicals, in low doses and in appropriate mixtures, can be helpful for the health of some animals. Often, the limits between nutrients, drugs and toxins are determined by the dose ingested [77]. A recent hypothesis on self-medication behaviour in animals considers the increase in the consumption of species already present in the usual diet [78], in contrast to the traditional hypothesis that considered the ingestion of specific compounds by animals only in response to infection [76]. Detailed field or laboratory observations are required to shed light about this possibility, buth such studies will represent a difficult if not impossible task with great bustards due to legal restrictions on their capture and handling, and problems to keep them as experimental animals in captivity.

Methods

Plant collection

Flowering *Papaver rhoeas* and *Echium plantagineum* plants were collected in May 2019 during the mating season, near Valdetorres del Jarama (Spain) and at one of the largest great bustard leks of central Spain (Fig. 2, UTM: 40.708987, -3.495657)[79–82]. These plant species were chosen based on their positive selection by great bustards (Fig. 1) and their potential toxicity as reported in the literature [37, see Discussion].

Plant extracts and essential oils

The plant parts were separated (flowers, leaves and capsules in the case of *P. rhoeas*), and dried at 40 °C during 48 h. Ground (40 g) flowers were extracted with methanol; and 50 and 30 g of ground leaves and capsules were sequentially extracted with hexane, ethyl acetate and ethanol in a Soxhlet apparatus. The solvents were evaporated under vacuum to give dry extracts.

Essential oils were obtained by hydrodistillation for 2 h in a Clevenger-type apparatus according to the European Pharmacopoeia. Ground flowers (100 g) were distilled with 2 l of water. The residual water (infusion) was freeze-dried (100 ml) or subjected to a liquid-liquid extraction (150 ml) with dichloromethane (DCM) (l50 ml x 3 times).

Antiprotozoal activity

T. gallinae trophozoites isolated from a common wood pigeon (*Columba palumbus*) from Central Spain were employed to determine the antiprotozoal activity. The strain was maintained by serial passes in 10 ml tubes with TYM medium. The tests were carried out 48 hours after a serial pass when trophozoites were at exponential growth phase. One hundred and fifty µl of a culture containing 500,000 trophozoites/ml were placed in each well of microtiter flat-bottom plates. Extracts and EOs were tested at several concentrations (400, 200, and 100 µg/ml) dissolved in 2 µl DMSO. After 24 h incubation at 37 °C the antiprotozoal activity was obtained by the MTT colourimetric method. Briefly, plates were centrifuged and the TYM was carefully removed. Ten µL of MTT/PMS (1.25 mg/ml; 0.1 mg PMS) were added to each well. After incubation for 15 min to occur the reduction of MTT, 50 µl sodium dodecyl sulphate (10 g SDS; 31,5 µl HCL; 100 ml distilled water) were added to dissolve formazan crystals obtained as a result of the reduction of MTT. Once the crystals had dissolved (15–30 min), the plate was read on a spectrophotometer at 620 nm. The activity was calculated as percentage growth inhibition (%GI) as follows:

$$\%GI = 100 - [(At - Ab)/(Ac - Ab)] \times 100$$

where *At* is the absorbance of treated wells, *Ac* the absorbance of control wells (not treated) and *Ab* the absorbance of blank wells (culture medium and vehicle only). All assays were carried out in triplicate and were repeated at least three times independently to confirm the results.

In vitro nematicidal effects

The nematodes used to determine anthelminthic activity came from a population of *Meloidogyne javanica* maintained on *Solanum lycopersicum* plants (var. Marmande) in pot cultures at 25ºC and 70% RH. The bioassays were carried out as described by Andrés et al. [83]. Freeze-dried infusions were dissolved in distilled water at concentrations adjusted to water-infusion yield (27 and 8 mg/ml for *P.*

rhoeas and *E. plantagineum*). Nematode inoculum (500 J2 in water) was filtered (25 µm), and the nematodes were suspended in 500 µL of lyophilized infusion treatments. Four aliquots (100 µL) of the nematode suspension (approximately 100 J2) and controls (water) were placed in 96-well plates. Organic extracts treatments were prepared by dilution in a DMSO-Tween solution (0.5% Tween 20 in DMSO) at 20 mg/ml and 5 µL of this solution were added to 95 µL of water containing 90–150 nematodes (final concentration of 1 mg/mL and 5% DMSO). Treatments were replicated four times. As a control, four wells were treated with the water/DMSO/Tween 20 in the same volume as the tests. The plates were covered to prevent evaporation and were maintained in the dark at 25 °C. After 24, 48, and 72 h, the dead J2 were counted under a binocular microscope. The nematicidal activity data are presented as percent dead J2s corrected according to Schneider-Orelli's formula [84].

Antifungal activity

Aspergillus niger (donated by Dr. K. Leiss, Wageningen University) was used to determine de antifungal activity [85].

Mycelium growth inhibition: *A. niger* was maintained in Petri dishes with PDA medium. The tests were carried out 24 hours after pass when the spores were not yet observed. Two ml of PDA mixed with 40 µl of each extract/EO suspended in ethanol and 10 µl of MTT at 5 mg/ml were filled in 12 well plates. The final concentration of extract/EO in the well was 1 mg/ml. Each test was performed in quadruplicate. Once the agar had solidified, the mycelium was inoculated by pitting. The plates were maintained in the dark at 28 °C for 48 h. Finally, the plates were scanned for reading and the diameter of the colonies was measured with the Image-J program. The percentage of mycelium inhibition was calculated according to Scheider-Orelli´s formula:

Mycelium inhibition=((*k*-*b*)/*k*)*100

where *b* is the average of diameters of the treatment replicas and *k* the average of diameters of the control replicas.

Spore growth inhibition

To obtain the spores, 5 ml of saline solution were added in three-day PDA Petri dish of *A. niger* culture. The spores were resuspended in saline solution with a sterile swab and filtered to remove the mycelium. Extracts and EOs were dissolved in 1% DMSO at a final concentration of 800 µg/ml in each well of a 96-well microplate. Each well contained 20 µl of spores (at a concentration of 7.5×10^5 spores/ml), 100 µl of RPMI medium and 160 µl of extract. The assays were made in quadruplicate. After 48 h incubation at 30 °C, the plates were revealed using the MTT colorimetric method. Briefly, 25 µl of RPMI with 5 mg/ml of MTT and 1 mg of menadione were added to each well and incubated 3 h in the dark. The medium was removed and 200 µl of 5% isopropanol in HCl (1M) were added. After 30 min of incubation at room

temperature and gentle agitation, the optical density (OD) was measured at 490 nm. For the calculation of the percentage of germination inhibition, the Scheider-Orelli´s formula adapted was used

% Germination inhibition = 100-((*b*/*k*)*100)

where *b* is the average absorbance of the treatment and *k* the average absorbance of the control.

Chemical analyses

The essential oils and hexane extracts where analyzed by gas chromatography mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 µl) were carried out by an AOC-20i and equipped with a 30 m × 0.25 mm i.d. capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimetil-(5%) diphenylpolisiloxane. Working conditions were as follows: split ratio (20:1), injector temperature 300ºC, temperature of the transfer line connected to the mass spectrometer 250 °C, initial column temperature 100 °C, then heated to 290 °C at 7 °C/min. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those found in the Wiley 229 and NIST Mass Spectral Database.

The alcoholic extracts (EtOH and MeOH) and aqueous extracts (infusions) where analyzed by liquid chromatography coupled to mass spectrometry (HPLC-MS) in a Shimadzu apparatus equipped with LC-20AD pump and a CTO-10AS VP column oven coupled to a mass spectrometer with simple quadrupole as analyzer (LCMS-2020 QP), with an electrospray ionization source (ESI). An ACE 3 C18 column (150 mm × 4.6 mm, 3 µm particle size) with an ACE3 C18 analytical pre-column was used for the separation. The compounds were eluted with Methanol (LC-MS grade) (MeOH) : MiliQ water with 1% acetic acid starting in isocratic method 10%MeOH during 5 minutes to continue with a gradient 10:67% MeOH during 20 minutes, 67:100% MeOH during 10 minutes, 100% MeOH during 10 minutes and 100:10% MeOH during 8 minutes, with a flow rate of 0.5 mL/min. The nitrogen flow (drying gas for solvent evaporation) was 15L/min. The potential for the electrospray capillary was + 4.50 kV and a Full Scan was used in positive mode (m/z 100–700) with a potential of 1.40 kV and a capillary temperature of 250ºC. The stock solutions of the extracts were injected at 0.25 mg/ml with a 5 µl injection through an automatic injector (SIL-20A XR). All extracts were dissolved in 100% MeOH for injection.

Statistical analyses of plants abundance in fecal samples

The occurrence of *Papaver rhoeas* and *Echium plantagineum* was calculated as the percentage of the total dry weight in faeces of males and females in a previous study [17]. The sex effect in diet was calculated in that study a whole, including all plant species. Here we calculated generalized linear mixed models (GLMMs, binomial error, logit link function) for *P. rhoeas* and *E. plantagineum* consumption by great bustards, which is a new statistical analysis. Fecal samples were collected at nine different sites

within the study area [17], but in the present study they were grouped for analysis in just two seasons: mating (April) and non-mating (November - January and July, respectively pre-mating and post-mating in [17]). Since the nine sites differed in availability of *P. rhoeas* and *E. plantagineum*, collecting site was included as a random factor in GLMMs. Likelihood-ratio tests were used to assess the significance effect of sex. Sample sizes were 81 female and 97 male faeces during the mating season, and 222 female and 223 male faeces during the non-mating season. We tested the effect of sex on the proportional consumption of each plant in fecal samples. The GLMM was repeated in the non-mating season as a complementary control of the main hypothesis: during mating males could eat both plants in higher proportions than females to counteract their higher parasite load due to stress, whereas in the non-mating season this sex difference could be smaller or even statistically non significant.

Abbreviations

Hex: Hexane

EtOAc: Ethyl acetate

EtOH: Ethanol

MeOH: Methanol

EO: Essential oil

IFD: Infusión-freeze dried

DCM: Dichloromethane

IDCM: Infusion-dichloromethane

L-Hex: Leaves-hexane

L-EtOAc: Leaves-Ethyl acetate

L-EtOH: Leaves-Ethanol

F-MeOH: Flowers-Methanol

F-IFD: Flowers-Infusión-freeze dried

F-IDCM: Flowers-Infusion-dichloromethane

C-Hex: Capsules-Hexane

C-EtOAc: Capsules-Ethyl acetate

C-EtOH: Capsules-Ethanol

GC-MS: Gas chromatography mass spectrometry

HPLC-MS: Chromatography coupled to mass spectrometry

PA: Pyrrolicidine alkaloids

J2: Second stage juvenile nematode

GLMM: Generalized linear mixed model

Pr: *Papaver rhoeas*

Ep: *Echium plantagineum*

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

AGC, JCA, LMB conceived the study; AGC, MTG, RAM and MFA designed the methods; PB and LMB collected plant samples; PB and MTG performed the experiments; PB, AGC, MTG, RAM and MFA analyzed the data; AGC, LMB and JCA contributed substantial resources and funding. PB, RAM, MTG and AGC wrote the manuscript’s first draft, and all authors contributed to subsequent editions. All authors discussed the experiments and results and their interpretation.

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Figures

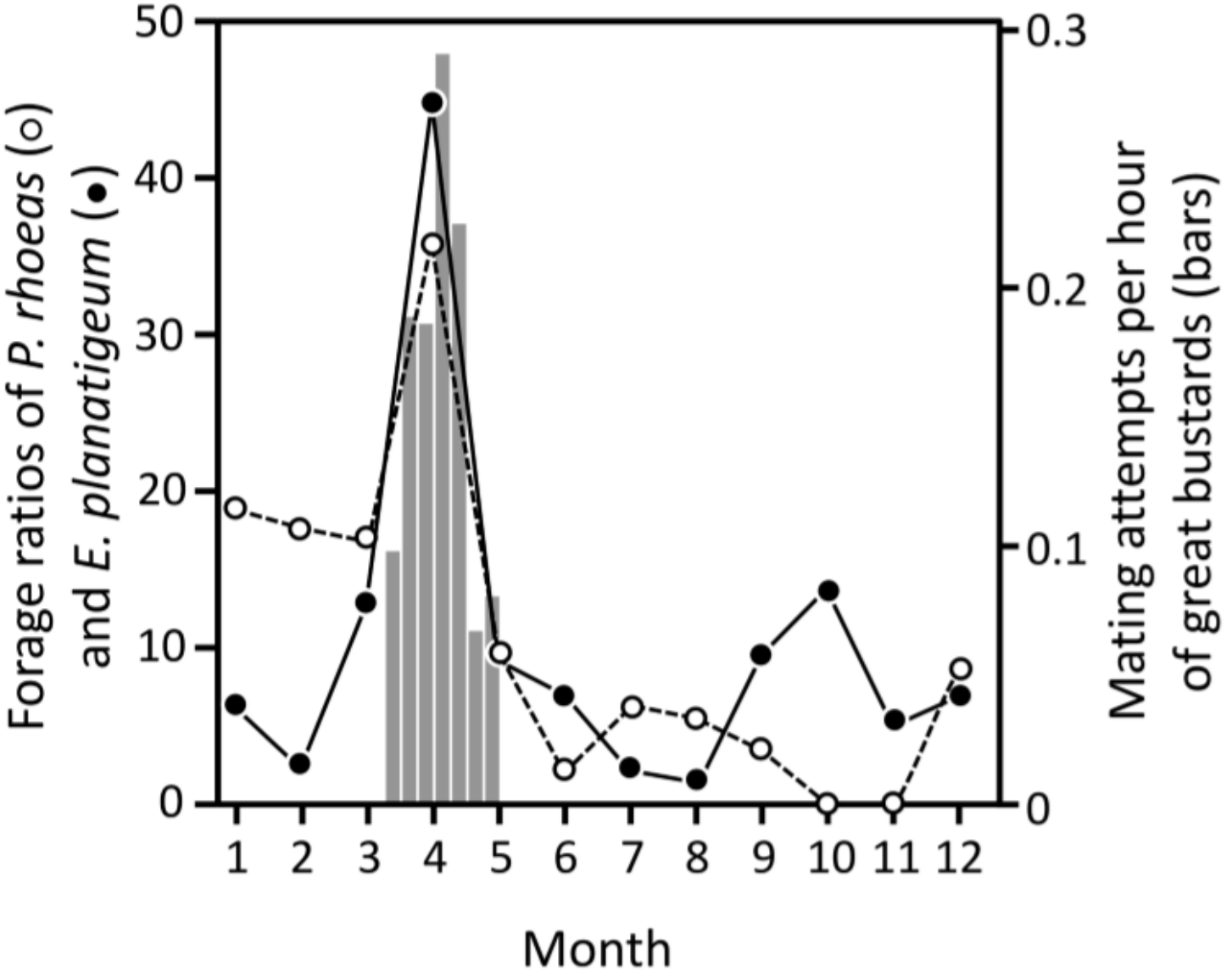

Figure 1

Monthly foraging ratios [18] of corn poppy (P. rhoeas, open circles) and purple viper's bugloss (E. plantagineum, filled circles) peaked in April, coinciding with maximum mating activity of great bustards (vertical bars, right axis). Figure composed with plant consumption and availability [16], and weekly mating attempts [86]. Vertical bars represent the number of mating attempts, defined as males full-displaying in very close proximity to one or more females (<3 m) with at least one of them showing obvious precopulatory behavior, independently of whether these attempts were followed by effective copulations or not. The number of mating attempts is a reliable indicator of mating success, as indicated by the significant correlation between rates of effective copulations and copulation attempts [19].

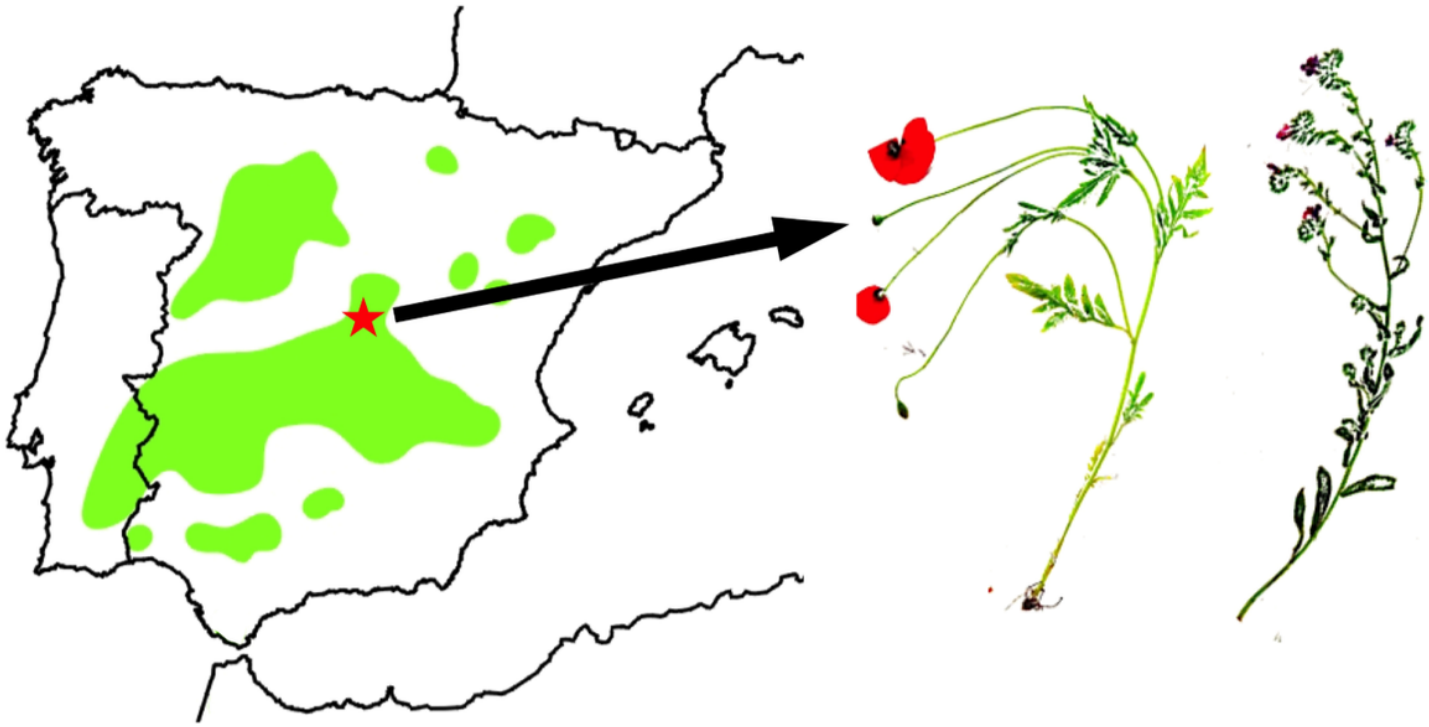

Figure 2

Great Bustard distribution in the Iberian Peninsula (green areas, see [87]). E. plantagineum (right) and P. rhoeas (middle) were collected in central Spain (red star: Valdetorres del Jarama, Madrid) within the Great Bustard area distribution