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# Allergenicity of Artemisia contained in bee pollen is proportional to its mass

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## KEY WORDS

Allergenicity; Artemisia pollen; bee pollen; biological potency; melissopalynology.

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## Summary

Bee product mugwort is identified as being at the origin of allergic accidents but the biological potency of Artemisia contained in bee pollen is not well known. In this experiment, Artemisia mass was identified in bee pollen mass and after having calculated the proportion of Artemisia using the bee pollen melissopalynology spectrum. Skin reactivity to Artemisia was assessed by measuring wheal diameters ( $W$ ) from skin prick tests using three serial dilutions of bee pollen on 11 allergic patients to Artemisia, in order to calculate the relationship between Artemisia mass ( $Mass_{artemisia}$ ) in bee pollen and skin reactivity.

The dose-response power regression curve ( $W_{artemisia} = 3.328 (Mass_{artemisia})^{0.297}$  ( $R^2 = 0.9947$ )) and the linear function  $Log_{10}(W_{artemisia}) = 0.297 (Log_{10}(Mass_{artemisia})) + 0.520$  ( $R = 0.9974$ )) were established using a bee pollen sample with 0.246 mg of Artemisia pollen per mg. Mugwort allergens seem to be little or not altered by bee secretions and bee pollen retains its allergenic capacity.

To our knowledge this is the first time it has been shown that skin reactivity of patients allergic to mugwort is proportional to the absolute mugwort mass contained in the bee pollen.

## Introduction

Pollen is flower sperm. It is the only source of certain macronutrients collected by worker honeybees. Collected from floral anthers at the tips of stamens, flower pollen grains stick to bee secretions. They are then assembled by the bee in loads placed in the baskets of the hind legs of the insect. Each load has a weight of 5 to 10 mg (1) and has several hundred thousand grains of a single floral species. Each load requires a visit to at least 80 flowers of the same plant type. The mixture of floral pollen into pellets is what is commonly called "bee pollen". It consists of various loads from different plant species.

Bees visit numerous plant flowers. There are, for example, more than 268 species and varieties of plants in England (2). G. Ricciardelli D'Albore and F. Intoppa have listed of all families of plants in Europe visited by bees (3).

Some floral pollen in bee products is responsible for allergies. Anaphylactic accidents related to the use of bee products are on the increase. There is substantial literature supporting this observation (4,5,6).

But, mugwort pollen and, more generally, the Asteraceae family are implicated in the origin of such accidents (7). A small proportion of Artemisia pollen in beehive products (only a few percent) is, however, enough to cause allergic symptoms. Asteraceae mainly includes genera Achillea, Artemisia, Carduus, Cichorium, Cirsium, Solidago and Taraxacum.

Artemisia's allergenic proteins appear to retain their allergenic properties in bee pollens from the time they enter the beehive to the harvesting by the beekeeper and their use by the end consumer (8).

To our knowledge, however, there is currently no technical definition of the allergenic potential of Artemisia in bee pollen. The

purpose of this study is to define the biological potency of Artemisia pollen in bee pollen *in vivo* by skin prick tests on patients allergic to Artemisia pollen.

## Material and Methods

### 1) Analysis of bee pollen spectrum

A pollen analysis of bee products is usually performed by a specialist laboratory by analyzing the beehive products. In our case, we used Honey Expertise Laboratory (Naturalim France Miel, 39330 Port-Lesney, France). Such analysis defines the type and frequency of each botanical genera or family floral pollen and determines the total mass of floral pollen. Bee pollen is thoroughly cleaned when intended for human consumption. Cleaning the pollen separates pollen dust balls, bee body fragments, wax or propolis. Melissopalynology is based on the European Maurizio and Louveaux standard without acetolysis, as recommended by the International Commission for Bee Botany (9). Counting and identifying floral pollen grains are made by examination under a microscope. Identification of pollen types is based on the laboratory pollen reference collection for local plants or on guides specialized in the pollen morphology of floral species.

Ten grams of well-homogenized bee pollen were dissolved and washed in distilled water, centrifuged, then prepared using aqueous glycerine and paraffin for smear preparations. Each smear was studied under a microscope to identify 500 floral pollen grains, in order to determine the percentage of each type of flower pollen. With bee pollen, the floral pollen mass is equated with the bee pollen mass, because it is accepted that the bee pollen pellets only contain kneaded floral pollen grains.

2) Calculation of the floral pollen allergen mass  $Mass_{p\text{-allergen}}$  in bee pollen is as follows:

2.1) Calculate the volume “Vpn” of each of the 1 to n types of floral pollen from the bee pollen spectrum using the formula  $V_{pn} = 4/3 \pi r^3$  if the pollen grain is spherical or using the formula  $V_{pn} = 4/3 \pi e^2 l$  if the floral pollen has an ellipsoidal shape.

The values of the radius  $r$  and of the mid-equatorial and longitudinal axes  $e$  and  $l$  are obtained from the literature from observations made on bee product pollen, including bee pollen (2). It is important to take into account changes in volumes of flower pollen due to orthodox or recalcitrant pollen qualities when pollen grains come into contact with aqueous bee fluids.

2.2) Calculate the proportion of volume  $P_{p\text{-allergene}}$  of flower pollen allergen «p-allergene»

$$P_{p\text{-allergene}} = \frac{(V_{p\text{-allergene}} \times \%_{p\text{-allergene}})}{\%_{p_2} + \dots + (V_{pn} \times \%_{pn})} / ((V_{p\text{-allergene}} \times \%_{p\text{-allergene}}) + (V_{p_2} \times \%_{p_2}) + \dots + (V_{pn} \times \%_{pn}))$$

(%pn is the percentage of flower pollen pn observed in the bee pollen analysis).

2.3) Calculate the mass of floral pollen allergen  $Mass_{p\text{-allergen}}$

$$Mass_{p\text{-allergen}} = P_{p\text{-allergen}} \times Mass_{pollens}$$

3) Calculation using the equation defining the allergenic potential of flower pollen allergen in bee pollen

Before applying this equation, it is necessary to:

- Use bee pollen with only one floral pollen allergen,
- Calculate the mass of floral pollen allergen as indicated above,
- Use a bee pollen without any floral pollen allergen as a “bee pollen negative control” to eliminate a skin sensitization to bee specific allergens.

3.1) Preparation of bee pollen extracts. Samples were prepared with the two types of bee pollen defined above. Five grams of fresh or frozen bee pollen was well homogenized on a glass plate and 450 mg of bee pollen was diluted in 4.5 ml of isotonic 0.4% phenol diluent (dilution weight/volume: 100 mg of bee pollen/ml of diluent) and was homogenized with a stirrer at a maximum speed for one minute. Samples were stored at room temperature for 24 hours and homogenized one more time with the stirrer before being removed, then 0.5 ml of the 100 mg/ml diluted solution was diluted in 4.5 ml of isotonic 0.4% phenol diluent to produce a 10 mg/ml diluted solution. These steps were repeated one more time to produce at least three samples of bee pollen, i.e. 100 mg/ml, 10 mg/ml and 1 mg/ml.

The allergen pollen floral mass contained per milliliter of each sample was deduced using the mass of floral pollen allergen in the bee pollen. Samples were kept at 5°C and were used within five days.

3.2) Measurement of skin reactivity to floral pollen allergen contained in bee pollen. Skin prick tests were duplicated on the inner side of the forearms of 11 subjects. Patients (seven women/four men) aged between 19 and 46 (mean: 29.7), who had been referred for seasonal symptoms (rhino-conjunctivitis and/or asthma) produced in July and August, were recruited in Hyères, in the south of France. They were not hyposensitized and were positive skin prick tested with a commercially available Artemisia vulgaris extract (Stallergenes) and sensitized to Art-v1 by testing for specific IgE-antibodies (> 0,27 kui/l). In addition to mugwort, they were sensitive to grasses (5), cats (3), cypress (4), olive (2), mites (7) and fungi mould (1) but none had a history of bee sting reactions. Informed consent was obtained from each patient.

Skin reactivity was assessed by geometric measuring of the two largest wheal diameters observed twenty minutes after the pollen sample prick tests, positive (histamine 10 mg/ml) and negative (glycerinated saline) controls and commercial extract tests (Stallergenes *Artemisia vulgaris* 100 IR/ml).  $W_{p\text{-allergen}}$  was defined by geometric measuring of skin reactivity to floral pollen allergen contained in bee pollen.

3.3) Analysis of the relationship between skin reactivity to floral pollen allergen in bee pollen  $W_{p\text{-allergen}}$  and floral pollen allergen mass  $Mass_{p\text{-allergen}}$ . If the model curve was a power regression.

$$(W_{p\text{-allergen}}) = b (Mass_{p\text{-allergen}})^a$$

then the linear function was calculated as follows:

$$\text{Log}_{10}(W_{p\text{-allergen}}) = a (\text{Log}_{10}(Mass_{p\text{-allergen}})) + B$$

where A and B are specific pollen allergen constants.

Variances analysis was performed by calculating  $R^2$ , which records the results of the value dispersions associated with regression. The closer  $R^2$  is to 1, the more the total variance is explained by the linear regression.

## Results

### 1) Calculation of $Mass_{artemisia}$ of bee pollen

Our bee pollen has a floral pollen allergen: *Artemisia*. It was collected in August 2012 in Eguisheim (France) at the GPS location: X 48.0428, Y 7.3062. Its spectrum includes 43.1% *Artemisia*, 25.7% *Mercurialis*, 16.0% *Lythrum salicaria* and 14.7% *Brassicaceae* (< 0,5% undetermined). *Artemisia*, *Mercurialis* and *Brassicaceae* are spherical pollens. Their respective diameters are 20, 25 and 20 micrometers. *Lythrum* pollen is ellipsoid in shape, the equatorial and longitudinal axes are respectively 26 and 36 microns. Indeterminate fractions were ignored.

#### 1.1) Calculation of $V_{pn}$ volumes

$$\text{Artemisia. } V_{artemisia} = 4/3 \pi (20/2)^3 = 4187 \mu^3$$

$$\text{Mercurialis. } V_{mercurialis} = 4/3 \pi (25/2)^3 = 8177 \mu^3$$

$$\text{Lythrum. } V_{lythrum} = 4/3 \pi (26/2) \cdot (36/2) \cdot 2 = 17643 \mu^3$$

$$\text{Brassicaceae. } V_{brassicaceae} = 4/3 \pi (20/2)^3 = 4187 \mu^3$$

#### 1.2) Calculation of $P_{artemisia}$ proportion

$$P_{artemisia} = (V_{artemisia} \times \%_{artemisia}) / ((V_{artemisia} \times \%_{artemisia}) + (V_{mercurialis} \times \%_{mercurialis}) + (V_{lythrum} \times \%_{lythrum}) + (V_{brassicaceae} \times \%_{brassicaceae})) = (4187 \times 43.1 \%) / ((4187 \times 43.1 \%) + (8177 \times 25.7 \%) + (17643 \times 16.0 \%) + (4187 \times 14.7 \%)) = 180460 / 734446 = 0.246$$

#### 1.3) Calculation of $Mass_{artemisia}$

$$Mass_{artemisia} = P_{artemisia} \times Mass_{pollens} = 0.246 \times 1 \text{ mg} = 0.246 \text{ mg}$$

There was 0.246 mg of *Artemisia* pollen per mg of bee pollen.

### 2) Calculation of $Mass_{hedera helix}$ of bee pollen

Our bee pollen is a pure, unique, floral pollen, *Hedera Helix* (99%; indeterminate percentage < 0,9%). It was collected in September 2013 in Thezillieu (France) at GPS location. X 45.8833, Y 5.6. This is a spherical pollen with a diameter of 25 micrometers.

#### 2.1) Calculation of volume $V_{pn}$

$$\text{Hedera helix. } V_{hedera helix} = 4/3 \pi (25/2)^3 = 8177 \mu^3$$

#### 2.2) Calculation of proportion $P_{hedera helix}$

$$P_{hedera helix} = (V_{hedera helix} \times \%_{hedera helix}) / (V_{hedera helix} \times \%_{hedera helix}) = (8177 \times 99\%) / (8177 \times 99\%) = 1$$

#### 2.3) Calculation of $Mass_{hedera helix}$

$$Mass_{hedera helix} = P_{hedera helix} \times Mass_{pollens} = 1 \times 1 \text{ mg} = 1 \text{ mg}$$

There was 1 mg of *Hedera helix* pollen per mg of bee pollen.

### 3) Measurements of skin reactivity to *Artemisia* and *Hedera helix* pollen and analysis of the relationship between $W_{p\text{-allergen}}$ and $Mass_{p\text{-allergen}}$

Out of the 11 patients sensitized to *Artemisia*, one was excluded because of a positive control test of less than 3 mm. Skin prick test results with three 10-fold dilutions of bee pollen with 0.246 mg of *Artemisia* pollen per milligram or with 1 mg of *Hedera helix* pollen per milligram are shown in **table 1**.

The model dose-response curve of *Artemisia* bee pollen is a power regression.

$$(W_{artemisia}) = 3.328 (Mass_{artemisia})^{0.297} \quad R^2 = 0.9947$$

The dose-response curve power regression is shown in **figure 1** and the linear function is:

$$\text{Log}_{10}(W_{artemisia}) = 0.297 (\text{Log}_{10}(Mass_{artemisia})) + 0.520 \quad R = 0.9974$$

The dose-response curve linear function is shown in **figure 2**.

The model dose-response curve of *Hedera helix* bee pollen is not a power regression.

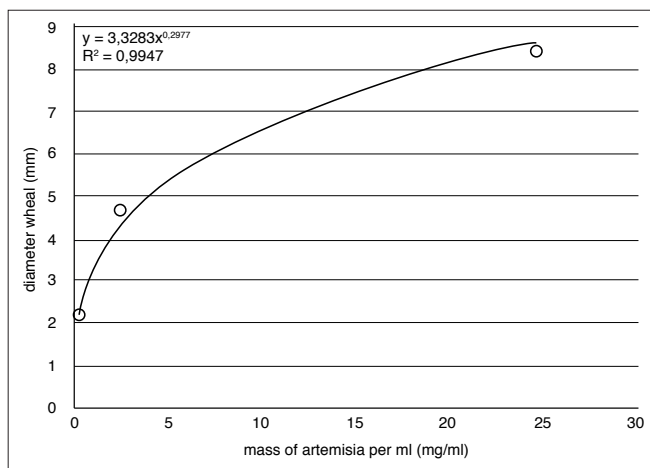
$$(W_{hedera helix}) = 0.27 (Mass_{hedera helix})^{0.033} \quad R^2 = 0.0292$$

**Table 1** - Skin prick test results with three 10 fold dilution of bee pollen with 0.246 mg of artemisia pollen per milligram or with 1 mg of hedera helix pollen per milligram.

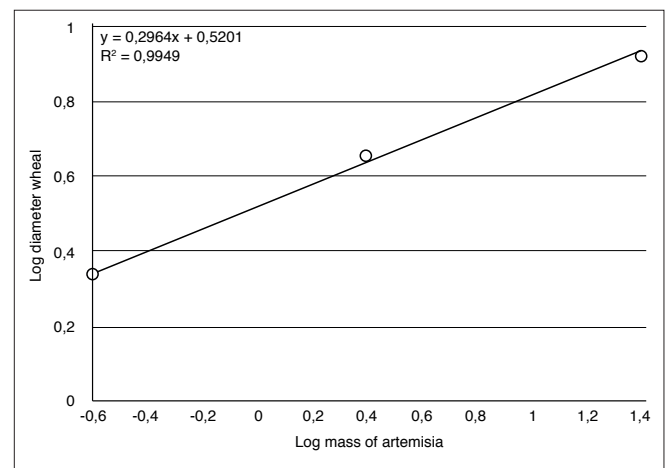
Patient	ARTEMISIA <sup>1</sup>				CONTROL <sup>1</sup>		HEDERA HELIX <sup>2</sup>		
	Artemisia 24.6 mg/ ml	Artemisia 2.46 mg/ ml	Artemisia 0.25 mg/ ml	Artemisia commer- cial extract	Positive control	Negative control	Hedera helix 100 mg/ml	Hedera helix 10 mg/ml	Hedera helix 1 mg/ml
P1	22.97	11	8.94	8.48	6.48	0	0	0	0
P2	4.90	2.83	1.73	4.9	7.93	0	0.5	0	0
P3	6.93	3.87	1	7.93	6.48	0	0.5	0.5	0
P4	11.96	9	4.90	9.48	3.46	0	0	0	0.5
P5	17.97	6	1.41	10.48	6.48	0	0	1	1
P6	6.93	1.73	1	3	4.24	0	0.5	0	1
P7	8	5.92	3.87	3.87	3.46	0	1	0	0.5
P8	8.94	6	2.83	9.38	3.87	0	0	0	0
P9	6.93	3.87	1.73	3.87	8.48	0	0	0	0
P10	3	2.83	1	3.87	6.92	0	0.5	0	0.5
Mean wheal	8.39	4.61	2.14	5.93	5.49	0	0.3	0.15	0.35

<sup>1</sup>geometric mean wheal (mm). <sup>2</sup>mean wheal (mm).

**Figure 1** - Dose-response curve power regression between  $W_{artemisia}$  and  $Mass_{artemisia}$



**Figure 2** - Dose-response curve linear function between  $log W_{artemisia}$  and  $Log Mass_{artemisia}$



## Discussion

Artemisia is one of plant species that provide bees with pollen but not nectar. Patients sensitized to Artemisia pollen who ingested bee products (honey, royal jelly, bee pollen) may experience an immediate allergic reaction because of cross-reaction

between Artemisia and Compositae bee product pollen and airborne Artemisia pollen (11,12,13,14,15).

However, patients who are allergic to bee products may be also sensitized to honeybee secretion proteins, pollen proteins contained in bee products (16) or bee venom components (7). This is why we tested our patients with bee pollen not containing

airborne pollen allergens, which was used as a bee pollen negative control. This was to eliminate skin sensitization to allergens other than *Artemisia* allergens (i.e., bee specific component allergens). Our bee pollen negative control was 100% *Hedera helix* bee pollen. *Hedera helix* pollen is not a common allergic pollen. In some rare cases, it might be responsible for cross-reaction to pollen panallergens (e.g., *olea*, *quercus*, *fraxinus*, *alnus* and *lolium*, but not *mugwort*) among Mexican allergic patients with dermatitis (17).

None of our patients had positive skin prick reactions to *Hedera helix* bee pollen. No relationship was established between *Hedera helix* bee pollen and skin reactivity.

A honeybee collects pollen grains at maturity from the male organs of flowers in order to obtain certain proteins or lipids. It gathers using an elaborate strategy based on pollen research of the highest quality for optimal protein and nutrient collecting. It takes advantage of the plant fertilization mechanisms in order to attain its objective, which is why the bee is not interested in wet pollen. As with floral nectar, wet pollen swells on contact with the secretions of sugar-water pollen grains that then release the soluble nutrient content. Based on comparisons between hand and bee-collected pollens, it appears that half or more of the mass of bee-collected pollens can be attributed to the addition of nectar-derived sugars to the pollen (18). The protein content of the grain decreases and this causes a leakage of the proteins in the external environment (19).

*It seems that our Artemisia bee pollen sample contains protein allergens that are exclusively Artemisia*

*Mercurialis*, *Brassicaceae* and *Lythrum* pollens were contained in our bee pollen sample. To our knowledge they are common in bee pollens. Literature searches in Medline were performed and no paper has described these pollens as being allergic pollens when they are included in bee pollens. This fact should be compared with what we know of the allergen qualities of these pollens.

- *Lythrum* is a strictly entomophilous pollen and is not known as an allergenic pollen.
- *Mercurialis* belongs to the *Euphorbiaceae* family. This family contains strongly sensitizing allergens (eg, latex, ricinus). *Mercurialis* allergens have shown allergenic cross-reactivity observed *in vitro* with profilins of other *Euphorbiaceae* and other families (e.g., *Oleaceae*, *Asteraceae*) (20), but the clinical significance is not well known (21). Furthermore, this cross-reactivity seems to be low (20) and the incidence of sensitivity to *Mercurialis* pollen is less than 0.9% among Italian patients with pollinosis (22).
- *Brassicaceae* pollen allergens are well known in cabbage, oilseed rape or mustard (e.g., profilin, calcium-binding protein, lipid transfer protein). They might be responsible

for cross-reactivity between foods and pollens (23,24), e.g. *mugwort*. The prevalence of sensitization of rapeseed pollen is correlated to exposure level and is higher (11.8%) among French atopic patients (25). In contrast, the prevalence of oilseed rape pollen allergy is very low (between 0.2% and 2%) in the United Kingdom, even in areas of high production (26,27), and the symptoms may be due to both allergens and irritant potentials of oilseed rape (28). In addition, our bee pollen was harvested in a vineyard monoculture area where there is no rapeseed or mustard cultivation and where the *Brassicaceae* genus, wild white rocket (*Diplotaxis*), is very common. The prevalence of sensitization to *Diplotaxis* pollen is low (14/410, i.e. 3.4%) and allergy even lower (3/410, i.e. 0.7%). It may be an occupational allergy in vineyard workers. In addition, as patients sensitized to *mugwort* do not report reactive symptoms to wild rocket pollen, there appears to be a biological cross-reactivity (29). In addition our patients are not winemakers.

Furthermore, there is no *Mercurialis*, *Brassicaceae* or *Lythrum* pollen in the analysis of the contents of the pollen traps of the French aerobiology network in the area neighboring to Hyères.

*It seems that our bee pollen sample with Artemisia contains Artemisia protein allergens*

In the literature, a strong correlation has been noted between cutaneous reactivity to bee pollen containing *mugwort* pollen and the cutaneous reactivity in patients with a positive skin prick test to an *Artemisia* commercial extract (8). Pitsios et al. found that approximately 40% of patients were sensitized to both bee pollen and floral *Artemisia* pollen. They considered that it might be due to *Asteraceae* pollen in their samples, which contained 20 mg of bee pollen per ml of solution.

This correlation was observed in their five bee pollen samples, but only two melissopalynology analyses of bee pollen samples have shown *Compositae* pollen. This might be due to the qualitative and quantitative methods used to analyse bee pollen. Only five spherules of different tinges were chosen from each bee pollen sample. Tinge loads are subjective. Colours change with time, if the loads are dry or are exposed to sunlight (30). Many plant species have pollen loads with very similar colours and sometimes up to three colours are observed for a single genus (2). *Compositae* pollen is often a minor bee pollen and choosing five pellets can raise the risk of non-homogenized samples.

On the contrary, our bee pollen was analyzed using the standard European melissopalynological method recommended by the International Commission for Bee Botany (9). This method is based on the study of 10 grams of well-homogenized bee pollen and 10 grams composed of more than 1.000 pellets. Our bee pollen sample is rich in *Artemisia* pollen, with 43.1% and 0.246



mg of Artemisia pollen per mg. Quantifying the absolute mass of Artemisia pollen with bee pollen per gram requires knowing the pollen spectrum of bee pollen and measuring pollen grain sizes. More particularly, this requires knowing pollen sizes when in contact with aqueous fluids. In contact with water, the pollen grain is in osmotic shock. Hydrated grain results in a change of its volume and opens pores and fissures (18) depending on the recalcitrance and orthodoxy of the pollen (31). Furthermore, two pollens of the same genus can have different reactions, e.g. Helianthus annuus pollen is orthodox and swells in contact with water, whereas Helianthus tuberosus pollen maintains the same volume (31).

A strong relationship was established between the absolute mass of mugwort pollen in bee pollen and skin reactivity despite our patient group including a small number of individuals sensitized to Artemisia and Art v1. The dose-response curve was a power regression curve:

$$(W_{\text{artemisia}}) = 3.328 (\text{Mass}_{\text{artemisia}})^{0.297} \quad (R^2 = 0.9947)$$

from which we were able to deduce the linear curve.

$$\text{Log}_{10}(W_{\text{artemisia}}) = 0.297 (\text{Log}_{10}(\text{Mass}_{\text{artemisia}})) + 0.520 \quad (R = 0.9974).$$

Mugwort allergens in bee pollen appears to be little or not altered by bee secretions and the allergens retain their allergenic capacity. In fact, the bee secretions contain digestive enzyme sugars (32) but are devoid of proteases. There is no protein digestion, as salivary and hypopharyngeal glands do not produce proteolytic enzymes (33).

## Conclusion

To our knowledge this is the first time it has been shown that the skin reactivity of patients who are allergic to mugwort is proportional to the absolute mugwort mass contained in bee pollen. Further studies are needed to determine how mugwort allergens retain their allergenic qualities.

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