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Challenging the safety and efficiency of homeopathy: investigating a step further for "placebo-like" effect on ants as model organisms

Abstract

Working on ants as biological models, we previously showed that Ignatia amara 5CH could decrease the adverse effect of onion odor (used as a stressing factor) on these insects' locomotion, tactile perception, social relationships, stress, cognition, learning and memory. We here examined if this beneficial effect may be due to a "placebo-like" effect, caused by the sugared compound used in homeopathic drugs. To do so, we studied, again on ants as model organisms, the effect of Ignatia amara Mk, the drug-free preparation of Ignatia amara homeopathic drug, on ants maintained in the presence of onion and thus impacted by its odor. The Ignatia amara Mk homeopathic product did not improve any of the physiological and ethological traits of ants affected by the stressing factor. As a control, we assessed all the examined traits in ants maintained under normal conditions and found that none of the considered traits was affected (i.e. was different from their usual valor, the ants being thus initially in good, usual health). Consequently, Ignatia amara Mk appears as being without any effects, while Ignatia amara 5CH appeared to be efficient as a drug. Therefore, it seems that the effect we observed in our previous study using Ignatia amara 5CH cannot be explained by the sugared compound used as a support for the 5 CH dilution of the drug. As ants are not affected by the placebo effect, this later being a psychological bias, our results suggest that our previous work indeed revealed some effects of Ignatia amara homeopathic drug.

Keywords: Cognition, locomotion, memory, *Myrmica sabuleti*, sensory perception, social relationships, stress

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Abbreviations: CH, Hundredth (CH) dilutions, also known as Hahnemanian Centesimal dilution; MK, Korsakovian dilution, 1000 time; ang.deg., angular degrees; ang.deg./cm, angular degrees per cm; mm/s, millimeter per second; χ^2, chi-square; *vs,* versus; n°, number; cm, centimeter; mm, millimeter; ml, milliliter; mg, milligram; µl, microliter; µg, microgram; s, second; min, minute; h, hour; t, time; %, percentage.

Introduction

Homeopathy is an alternative medicine, conceived in 1796 by Hahmann, based on the hypothesis that a substance causing symptoms can also cure persons suffering from such substance. To care of ill persons, very low concentrations of the adequate substance (i.e: the one causing the symptoms) are provided to the patient using diluted solutions. The dilution rate might be very high (each CH correspond to a 100 time dilution, so 10^{-2} of the initial concentration), and may reach 10^{-30}. Therefore, the most diluted solutions (below 12CH, so 10^{-24} of the initial concentration) may even no longer contain any molecule of the substance, and thus, according to the laws of chemistry, should have no effect, as no chemical reaction can occur in the absence of the reagents. Indeed, many meta-analyses have shown that homeopathy treatments have no valid effect.[1,2] Persons treated thanks to homeopathy may simply experience a placebo effect.[3-5] Several lands have removed funding for homeopathic remedies.

We have previously examined, on ants as model organisms, the effect of the homeopathic drug Ignatia amara 5CH (corresponding to a dilution of 10^{-10} of the initial concentration) on the stress induced in ants by onion,[6] and we found that, very unexpectedly, this drug reduced the ants' state of stress, as it is claimed to act on the drug notice. We were quite surprised by these results, as we expected to observe no statistically significant differences between ants under normal diet and ants under the drug diet. We therefore formulated two hypotheses for explaining our results. The first one postulated that our results were affected by the small size of our experimental samples, in terms of numbers of individual ants tested. However, we took the precaution to use non-parametric tests to investigate the significance of our results. As non-parametric tests are usually less powerful than parametric tests, this should protect us from detecting a significant effect where in fact there is none. The second hypothesis is that the sugared (sucrose/lactose) globules on which the active substance and the exipients were deposited are the cause of the observed changes in ants' behavior. We thus decided to test a somewhat called "placebo-like" effect of *Ignata amara* globules on ants, using the "placebo" version of that drug, i.e. globules without any deposited active substance or exipients, namely Ignatia amara Mk. Mk, or 1000K, stand for a 1000 time dilution, according to the Korsakov dilution technique. Therefore, a MK dilution rate is equivalent to a $0.01*1^{1000}$ dilution of the initial concentration. This number is so small – even in comparison with the Avogadro number (6.022^{+23}) which estimates the number of molecules in one mole of a substance - that we could without any doubt consider that a sample of Ignata amara MK does not contain any molecule of the active substance.

We thus intended to examine if Ignata amara MK drug may have a calming effect similar to that of the drug Ignatia amara 5CH. Briefly, we set ants in the presence of onion (the stress factor) and assessed as in our previous study several of their ethological and physiological traits, comparing our results with those obtained in our first work on

the subject. Then, we gave the homeopathic "placebo" Ignatia amara Mk to these ants living in the presence of onion, assessed their same traits and compared the results with those just before obtained for ants in presence of onion. After that, we relocated the ants in a new tray having never contained onion and we replaced their sugar water tubes by new ones not containing the homeopathic product. One day later, as a control, we again assessed the considered ants' traits and compared the obtained results with those just before obtained for ants having onion around them and consuming Ignatia Mk. These three series of experiments should reveal if the homeopathic "placebo" Ignatia Mk can decrease the ants' state of stress thanks to the sugared granules of the product.

Before relating our experimental work, we here below briefly recall the symptoms *Ignatia amara* is supposed to treat, why we use ants as models, which species we used and what we know on it, as well as the ethological and physiological traits we considered.

Information on Ignatia amara

Ignatia amara drug is produced from the bean of Saint Ignatius, the seed of a *Strychnos ignatii* P.J.Bergius, 1778, a species of small shrub belonging to the Loganiaceae family, native to the Philippines. This species was formerly called *Ignatia amara* and this name remained for the homeopathic drug produced from the plant extract. The seeds of this plant contain two alkaloids, strychnine and brucine. Ignata amara is recommended by homeopaths for treating, among others, anxiousness, insomnia, nervousness, dizziness, headaches, and nervous breakdowns, each time using an adequate dosage [https://www.doctissimo.fr › souches-homeopathiques, https://pharmacieetnature.com › produit › 1773-ignatia-amara, https://pharmacy-medi-market.be › pharmacie › ignatia...]. This is why we studied its effect on ants in presence of onion because this plant, due to its chemical compounds, largely stress, annoys and irritates them.

Why using ants as models?

Most important biological traits are identical for any animal species (genetic, muscles contraction, nerve impulses, respiration, protein synthesis …).[7] This explains why physiological, medicinal and ethological studies are firstly conducted in animals, and thereafter in humans.[8] For instance, fruit flies, cockroaches, bees, mice, and monkeys are often used. Insects are often used since they rapidly develop and can easily be maintained in laboratory.[9] Hymenoptera, among others, are used, and ants can thus be used.[10] They can be so the more because they detain complex behavior and several evolved skills. Among others, they build complex nest, communicate with congeners thanks to pheromones, mark the different parts of their territories, navigate using memorized cues, take care of their brood and queens, clean their nest and manage cemeteries at its frontiers.[11] The effect of substances and environmental factors on these abilities can thus be examined, what allows emitting hypothesis about the effects of these substances and factors on humans' health.

Which species was used and what is known on it?

We worked on the species *Myrmica sabuleti,* Meinert, 1861, the biology of which we now well know. Indeed, we studied their eyes morphology, visual perception, conditioning ability, recruitment strategy, navigation system, as well as the ontogenesis of some of their abilities.[12,13] They detain several numerosity abilities, can expect the occurrence of an event on the basis of previous such occurrences, recognize themselves in a mirror, to name just a few of their abilities.[14-17] We here once more used *M. sabuleti* as a model for studying the efficiency of a homeopathic placebo.

Which traits were considered?

We intended to examine the impact of onion as well as of onion with at the same time Ignatia Mk on the ants' food intake, general activity, locomotion, orientation ability, tactile (pain) perception, social relationships, stress, cognition, conditioning ability and memory. The experimental methods for doing so are identical to those many times used and explained in published papers.[18-20] They will be here rather briefly related, though not avoiding some inevitable plagiarism.

Materials and methods

Collection and maintenance of ants

The experiments were conducted on two colonies of *M. sabuleti* collected in May 2021, in the Aise valley (Ardenne, Belgium), in an abandoned quarry. The colonies contained about 600 workers, brood and a queen. Each colony was maintained in one to three glass tubes half filled with water, a cotton plug separating the water from the ants' compartment. The nest tubes of each colony were set in a tray (34cm x 23cm x 4cm) which served as a foraging area. In them, pieces of *Tenebrio molitor* larvae (Linnaeus, 1758) were provided three times per week, and a small cotton plugged tube filled of sugar water (concentration: 15%) was permanently provided (Figure 1). The luminosity of the laboratory equaled *ca* 330 lux while working on ants and *ca* 110 lux during the other time periods. The ambient temperature equaled *ca* 20°C, the humidity *ca* 80%, and the electromagnetic field *ca* 2 µWm2, all these conditions being suitable to the species. Ants are here often named 'workers' or 'nestmates' as commonly do researchers on social insects.

Figure 1 Successively, 1 to 6: the two used colony, the built of the cube used for conditioning ants, set of pieces of onion in the ants' foraging area, the homeopathic product here analyzed, the realization of the solution of this product given to the ants, and ants drinking this solution.

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Onion and solution of Ignatia amara Mk given to the ants

We deposited 16 pieces (about 1 cm^3) of onion on the two colonies' foraging area, between the nest entrances and the food sites, and we renewed these pieces of onion each two days (Figure 1). A package of Ignatia amara Mk , Boiron® (F 695 10 Messigny) was provided by the pharmacist Wera (1170 Bruxelles). According to their symptoms, humans are advised to consume one to five tablets of this homeopathic product each day. Humans treated with Ignatia amara Mk consume thus, meanly, 3 tablets of this product drinking at the same time about one liter of water. Insects, and thus ants, due to their anatomy (cuticle) and physiology (excretory apparatus), consume about 10 less water than mammals. Consequently, to provide the ants with an Ignatia amara Mk diet similar to that of humans, they must be provided with a solution of 3 tablets of this product in 100 ml of water. Due to the needed amount of solution for performing the experiments using it, and to the ants' need of sugar, we made a solution of 1 tablet in 33 ml of sugar water, that usually given to the ants. We delivered this solution to the ants in their usual sugar water tubes plugged with cotton. We checked each day if the ants consumed the provided solution, and effectively they did (Figure 1).

Food consumption, general activity

For each three conditions of ants' maintenance, the workers sighted on the meat food, those staying at the entrance of the sugar water tube, and those being active at any places (foraging area, food sites, inside of the nest) were counted four times for each two used colonies (n° of counts: 4 x 2 = 8 for each kind of count). The daily mean of these 8 counts was established for each kind of maintenance and each kind of count (Table 1, lines 1 to 6). For each kind of count, these six daily means, obtained for ants under one and another kind of maintenance, were compared using the non-parametric test of Wilcoxon.[21] In addition, for each kind of maintenance and of count, the mean of the six daily means was established (Table 1, last line).

Table 1 Effect of Ignatia Mk on the impairments caused by onion on the ants' food intake and general activity. For each trait and situation, the table gives the mean numbers of counted ants each day (lines I to VI) as well as the mean of these six daily means (line I-VI). The homeopathic product did not ameliorate any of the three considered traits affected by the onion odor

days	with onion			onion + Ignatia Mk			normal situation		
	meat	sugar water	activity	meat	sugar water	activity	meat	sugar water	activity
I	0.5	0.75	8.25	0.5	0.63	9.13	1	2	14.25
II	0.38	1	8.38	0.13	0.88	10.5	1.25	2.25	15.25
III	0.38	0.5	8	0.13	0.75	9	1.5	1.88	11.5
IV	0.38	0.38	6.5	0.25	0.63	8.5	1.63	2.63	16.25
V	1.5	1.38	10.5	1.13	1	8.5	1.5	2.5	17.88
VI	0.5	1.5	10	0.75	1	8	1.63	2.63	17.38
I-VI	0.6	0.75	8.6	0.48	0.82	8.94	1.13	2.32	15.42

Linear and angular speeds, orientation

These traits were assessed on ants freely moving in their foraging area, the speeds without stimulating them, the orientation while stimulating them with a nestmate tied to a piece of paper (Figure 2A). A tied nestmate emits its attractive mandible glands alarm pheromone. To assess the ants' speeds and later their orientation, 40 trajectories were recorded and analyzed thanks to software[22] set up according to the following definitions. The linear speed (in mm/s) was the length of a trajectory divided by the time spent to travel it; the angular speed (in ang.deg./cm) was the sum of the angles made by successive adjacent segment of a trajectory divided by the length of this trajectory; the orientation (in ang. deg.) to a location was the sum of successive angles made by the direction of the trajectory and the direction towards the location, divided by the number of measured angles. An orientation value lower than 90° signifies a tendency to walk toward the location. An orientation value larger than 90° signifies an avoidance of the location. For each quantified variables, the median and the quartiles of the 40 obtained values were established (Table 2, lines 1, 2, 3) and the distribution of these 40 values obtained for the three ants' maintenance conditions was compared to one another using the non-parametric χ^2 test.[21]

Table 2 Effect of Ignatia Mk on the impairments caused by onion on five ants' physiological and ethological traits. The table gives the median (and quartiles) or the mean [and the extremes] of the recorded data. The homeopathic product appeared to be ineffective at counteracting the adverse effects of onion odor. mm/s = millimeter per second, ang. deg. = angular degree; cm = centimeter, n° = number

Traits	with onion	onion + Ignatia Mk	normal situation
linear speed (mm/s)	12.4 (10.3 – 13.8)	11.5 (10.5 – 13.8)	9.6 (8.4 – 10.1)
angular speed (ang.deg./cm)	155 (142 – 171)	167 (148 – 180)	116 (102 – 140)
orientation (ang.deg.)	69.0 (58.6 – 78.9)	61.7 (52.8 – 81.7)	34.5 (25.4 – 44.1)
audacity (n°)	1.50 [0 – 2]	1.45 [1 – 2]	3.15 [2 – 4]
tactile (pain) perception			
linear speed	8.1 (7.5 – 8.6)	8.2 (7.3 – 8.8)	5.1 (4.7 – 6.0)
angular speed	138 (122 – 160)	145 (131 – 159)	279 (262 – 303)
on a rough substrate			

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Audacity

This trait was evaluated through the ants' tendency in coming onto an unknown apparatus provided in their foraging area. This apparatus consisted in a cylinder (height = 4 cm; diaeter = 1.5) vertically tied to a squared platform (9 cm^2), these two pieces being in white strong paper Steinbach® paper (Figure 2B). For each two colonies, the workers sighted at any place on the apparatus were counted 10 times over 10 minutes (n° of counts: 10 x 2 = 20). The mean and the extremes of the obtained numbers were established (Table 2, line 4). The successive numbers obtained for each two colonies were correspondingly added; then they were chronologically summed by two. This doing provided five successive sums, and those relative to each kind of ants' maintenance were compared to one another using the non-parametric Wilcoxon test.[21]

Tactile (pain) perception

If perceiving the rough character of a substrate, ants walk on it with difficulty, slowly, sinuously, often touching the substrate with their antennae. If poorly perceiving the uncomfortable character of a substrate, ants walk on it rather easily, rapidly, not very sinuously, and seldom touch it with their antennae. Therefore, for evaluating the ants' tactile (pain) perception, their linear and angular speeds of their walking on a rough substrate were assessed as explained in the subsection relative to these speeds assessment. This was done using an apparatus, for each colony, consisting of a tray (15 cm x 7 cm x 4.5 cm) inside of which a piece of n° 280 emery paper duly folded was set in order to divide the tray in a first 3 cm long zone, a second 3 cm long one containing the emery paper, and a last 9 cm long zone. An experiment consisted in setting 12 ants of each two colonies in the first zone of their own apparatus, and in recording their trajectories when they walked on the emery paper. The ants' linear and angular speeds were then assessed as usually, the number of analyzed trajectories equaling 24 (Figure 2C). For each kind of speed, the median and quartiles were established (Table 2, lines 5, 6), and the distributions of the 24 values obtained for each kind of ants' maintenance were compared to one another using the non-parametric χ^2 test.[21]

Brood caring

For each two colonies, a few larvae or nymphs were removed from the nest and set in front of the entrance. In each two colonies, five of these larvae or nymphs and the workers' behavior towards them were observed during five minutes (Figure 2D). The number (5 x 2 = 10) of these larvae or nymphs not re-entered after 30 seconds, 1, 2, 3, 4, and 5 minutes were counted (Table 3, line 1). Only ten larvae or nymphs were considered because all of them must be looked simultaneously. Also, the experiment was performed only once because removing brood out of the nest generates a strong social perturbation which could imperil the brood survival. The six successive numbers of not re-entered larvae or nymphs obtained for each two colonies were correspondingly added, and the six sums obtained for ants maintained under the different used experimental conditions were compared to one another using the non-parametric test of Wilcoxon.[21]

Table 3 Impact of Ignatia Mk on the stress induced by onion on four ants' physiological and ethological traits. The homeopathic product appeared to not change the ants' affected behavior presented in the presence of onion, i.e. it was inefficient. n° = number, aggressive levels are defined in the text, 'a' = numbers of levels 2 + 3 + 4 / 0 + 1, min = minute, s = second

Traits	with onion	onion + Ignatia Mk	normal situation
brood caring: n° of not reentered larvae over 5'	30s 1 2 3 4 5 min	30s 1 2 3 4 5 min	30s 1 2 3 4 5 min
	10 10 8 8 6 4	10 10 9 8 7 7	9 7 4 1 0 0
relationships: n° of aggressive levels 0-4;'a'	0 1 2 3 4 'a'	0 1 2 3 4 'a'	0 1 2 3 4 'a'
	23 48 56 2 0 0.82	20 36 53 0 0 0.95	56 50 13 0 0 0.12
escaping: n° of ants escaped over 12 min	2 4 6 8 10 12 min	2 4 6 8 10 12 min	2 4 6 8 10 12 min
	0 0 0 0 2 4	0 0 0 2 2 3	1 4 6 8 10 11
cognition: n° of ants in front (f) and beyond (b) a twists and turns path over 12 min	2 4 6 8 10 12min	2 4 6 8 10 12min	2 4 6 8 10 12min
	f 22 19 17 16 15 14	f 26 21 19 17 15 14	f 23 18 14 12 11 10
	b 0 0 0 0 1 2	b 0 0 0 0 2 3	b 0 1 2 3 6 9

Social interactions

Ants pertaining to the same colony do not aggress themselves. This social peaceful behavior may be affected by environmental elements or consumed products. The effect of onion and of onion together with the consumption of Ignatia Mk on these social interactions was studied by performing, for each kind of maintenance, five dyadic workers' encounters for each two used colonies. These 2 x 5 encounters were made in a cup (diameter = 2 cm, height = 1.6 cm), the borders of which having been covered with talc to prevent ants from escaping, and during each encounter, an ant of the pair was observed during 5 minutes. The numbers of times it did nothing (level 0 of aggressiveness), touched the other ant with its antennae (level 1), opened its mandibles (level 2), gripped and/or pulled the other ant (level 3), and tried to sting or stung the other ant (level 4) were recorded (Figure 2E, Table 3, line 2). The numbers obtained for the two colonies and the ten observed ants were correspondingly added. The distributions of the values obtained for the five levels of aggressiveness under the ants' three kinds of maintenance (onion, onion + Igantia M^k, normal situation) were compared to one another using the non-parametric χ^2 test.[21] In addition, each time, a variable 'a' evaluating the ants' social relationships was established, this 'a' variable being the number of aggressiveness levels 2 + 3 + 4 divided by that of aggressive levels 0 + 1 (Table 3, line 2).

Stress, cognition

In order to escape from an enclosure, an individual must stay calm, not stressing, and cautiously look for an exit. Also, it must detain

some intact cognitive abilities. To study the state of stress and the cognition of ants living under the three considered maintenances, each time, for each two colonies, six ants were enclosed under a reversed cup (made of polyacetate; height = 8 cm, bottom diameter = 7 cm, ceiling diameter = 5 cm) set in the foraging area. The inside surface of these enclosures were covered with talc to prevent ants from climbing on it. In the bottom rim of the enclosure, a notch (3 mm height, 2 mm broad) was managed as an exit allowing the ants escaping (Figure 2F). Twenty seconds after that the six ants were in the enclosure, those escaped after 2, 4, 6, 8, 10 and 12 minutes were counted for each two colonies. The numbers obtained for the two colonies were correspondingly added (Table 3, line 3). The six sums obtained for ants living under the three considered kinds of maintenance were compared to one another using the non-parametric Wilcoxon test.[21]

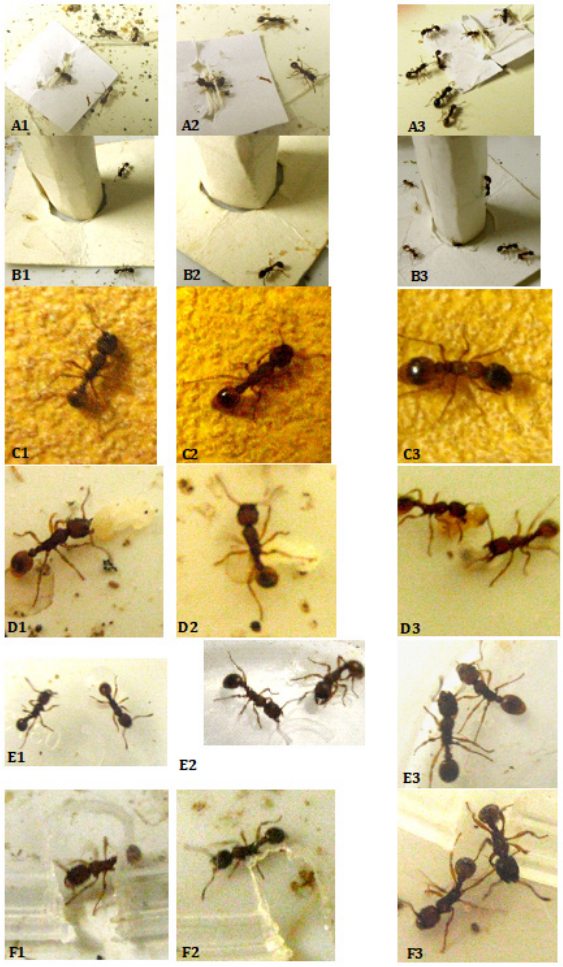

Figure 2 Some views of the experiments made to know the efficiency of Ignatia Mk. 1: pieces of onion on the ants' foraging area; 2: still with such pieces with in addition the homeopathic product in their sugar water; 3: normal maintenance, i.e. no onion, no homeopathic product. Obviously, the ants similarly and not as usual behaved under the two first maintenances, and correctly, normally under the third maintenance.

Cognition

For each considered maintenances, the ants' cognition was evaluated through their ability in crossing a twists and turns path. To do so, two pieces (each one measuring 4.5 cm x 12 cm) of strong white paper (Steinbach®) were duly folded and inserted in a tray (15 cm x 7 cm x 4.5 cm) what allowed obtaining a twists and turns path between a first 2cm long zone in front of this path, the difficult path, and a 8 cm long zone beyond the path (Figure 3A). Each colony had its own apparatus. An experiment on a colony consisted in transferring 15 workers into the first zone of the apparatus and in counting after 2, 4, 6, 8, 10 and 12 minutes the ants still in that zone as well as those having reached the zone located beyond the twists and turns path. The numbers obtained for the two colonies were correspondingly added (Table 3, line 4). For the zone lying in front and that lying beyond the difficult path, the six sums obtained for ants living under the three considered maintenances were compared to one another using the non-parametric Wilcoxon test.[21]

Learning, memory

For each two used colonies, at a given time, a green (maintenance with onion), yellow (maintenance with onion + Ignatia M^k), blue (normal maintenance) hollow cube (Figure 1), made of strong paper (Canson®), was set above the entrance of the tube filled of sugar water (Figure 3Ba) and the pieces of mealworm were in addition deposited very near the cube. The use of three different colors for the cube is due to the fact that, since an individual has acquired conditioning to a stimulus, it keeps its conditioning during a rather long time and can no longer be sued for assessing its conditioning to this stimulus. As soon as having deposited the adequate cube, the ants underwent operant visual conditioning. Over the ants' conditioning acquisition, then after the cube removal, over the ants' loss of conditioning, the workers of each two colonies were tested in a Y-maze, each colony having its own maze. The latter was made of strong white paper, had its sides slightly covered with talc, was deposited in a separated tray (15 cm ×7 cm × 5 cm), and was provided with an adequate hollow cube randomly in its left or right branch. To make a test on a colony, 10 workers were one by one deposited in the maze before its division into two branches, and the ants' choice of one or the other branch of the maze was recorded (Figure 3Bb). Choosing the branch containing the cube was giving the correct response. After a worker had been tested, it was kept in a glass until 10 ants of its colony were tested, and after having tested 10 ants, all of them were set back in their foraging area. Tests were performed 7, 24, 31, 48, 55 and 72 hours after the start of the ants' conditioning as well as after the cube removal (Table 4). The responses given by ants of the two colonies were correspondingly added, what allowed establishing the proportions of correct responses (the ants' conditioning scores) at each considered times (Table 4). The six + six proportions obtained for ants living under the three considered maintenances were compared to one another using the non-parametric Wilcoxon test.[21]

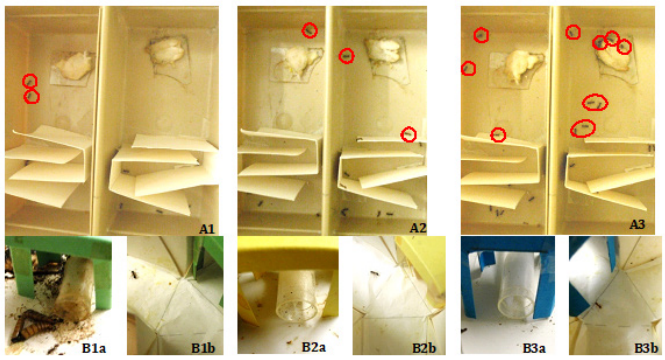

Figure 3 Photos of two experiments made to know if Ignatia Mk had a real effect. 1: pieces of onion in the ants' foraging area; 2: such pieces and Ignatia Mk in the ants' sugar water; 3: normal situation. A: experiment relative to the ants' cognition; Ba: ants' training, Bb: ants' test. Obviously, the homeopathic product did not change the ants' behavior observed in the presence of onion and impacted by this plant odor. It was inefficient.

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Table 4 Impact of Ignatia Mk on the effect of onion on the ants' ability in acquiring conditioning and on their memorization. The table gives the numbers of ants of two colonies giving the right *versus* wrong responses when tested in a Y maze, and on this basis, the ants' conditioning scores. The homeopathic product did not counteract the situation (poor conditioning acquisition) occurring in the presence of onion; it was inefficient

Time (hours)	with onion			onion + Ignatia Mk			normal situation		
7	5 vs 5	5 vs 5	50%	5 vs 5	4 vs 6	45%	6 vs 4	7 vs 3	65%
24	5 vs 5	6 vs 4	55%	5 vs 5	5 vs 5	50%	6 vs 4	7 vs 3	65%
31	3 vs 7	5 vs 5	40%	4 vs 6	5 vs 5	45%	7 vs 3	7 vs 3	70%
48	6 vs 5	5 vs 5	55%	5 vs 5	4 vs 6	45%	8 vs 2	8 vs 2	80%
55	5 vs 5	5 vs 5	50%	6 vs 4	6 vs 4	60%	8 vs 2	8 vs 2	80%
72	7 vs 3	6 vs 4	65%	7 vs 3	6 vs 4	65%	9 vs 1	9 vs 1	90%
cue removal									
7	6 vs 4	6 vs 4	60%	7 vs 3	5 vs 5	60%	9 vs 1	9 vs 1	90%
24	5 vs 5	6 vs 4	55%	6 vs 4	5 vs 5	55%	8 vs 2	9 vs 1	85%
31	6 vs 5	5 vs 5	55%	5 vs 5	5 vs 5	50%	9 vs 1	7 vs 3	80%
48	6 vs 4	6 vs 4	60%	6 vs 4	6 vs 4	60%	8 vs 2	8 vs 2	80%
55	5 vs 5	4 vs 6	45%	4 vs 6	6 vs 4	50%	8 vs 2	8 vs 2	80%
72	6 vs 4	4 vs 6	50%	6 vs 4	4 vs 6	50%	9 vs 1	8 vs 2	85%

Results and discussion

Though the experiments were made on ants successively maintained with pieces of onion on their foraging area, with such pieces of onion and the homeopathic product in their sugar water, and finally under normal conditions, the results are here below reported otherwise. The different ants' ethological and physiological examined traits are successively considered, and each time, the results obtained for the three kinds of ants' maintenance are related, referring to Tables and Figures which are presented exactly in the same order, all this for the readers' convenience.

Food intake, activity

Results are given in Table 1. With pieces of onion in their foraging area, the ants eat and drunk little, and were not very active at any time. When the homeopathic product was added to their sugar water, they behaved nearly in the same way. Though there was some differences due to the development of the brood (several larvae became nymphs and no longer needed meat), statistically, these differences were not significant: meat intake: N=5, T=+3, -12, P=0.156; sugar water consumption: N=6, T=+7, -14, P=0.281; activity: N=6, T=+13, -8, P=0.344. Ignatia Mk did not ameliorate the ants' food consumption and activity impacted by the odor of onion. In the absence of the latter plant and the homeopathic product, after having recovered for one day, the ants consumed more meat, drunk more sugar water, and were far more active than while living under the two previous experimental conditions. For the three examined traits, the difference was statistically different: N=6, T=21, P=0.016. The ants were thus initially in good health, but were affected by onion odor, and Ignatia Mk could definitely not reduce the adverse effect of onion. The latter product was thus inefficient, at least for the three here considered traits.

Linear and angular speeds

Results are given in Table 2, lines 1, 2. While having pieces of onion in their foraging area, the ants walked erratically, sinuously, quickly, often jerking, and obviously stressing. The obtained values of speeds (12.4 mm/s, 155 ang.deg./cm) were very similar to those previously obtained for ants under such a maintenance (12.8 mm/s, 157 ang.deg./cm).[6] While consuming in addition the homeopathic product, the ants' linear speed was statistically similar to that presented in only the presence of onion (χ^2=1.81, df=2, 0.30 < P < 0.50) while their angular speed was statistically somewhat larger (χ^2=7.51, df=2, 0.02 < P < 0.05). This could be due to an increase of the effect of onion (present near the ants since 12 days), and to an inefficiency of Ignatia Mk in reducing the perturbing and stressing effect of onion. With no onion on their foraging area and no Ignatia Mk at their disposal, the ants walked less rapidly and less sinuously, and this was statistically significant: linear speed: χ^2=26.68, df=2, P < 0.001; angular speed: χ^2=43.10, df=3, P < 0.001. Trajectories recording and assessment was repeated and the obtained results were similar: linear speed: 10.9 (9.9 – 11.0) mm/s; angular speed: 120 (100 – 123) ang.deg./cm. Consequently, the experimented ants, when normally maintained, walked as usually, i.e. not rapidly, not sinuously; onion increased these two speeds; and Ignatia Mk did not reduced the impact of onion on the ants' locomotion, i.e. it was inefficient.

Orientation

Results are given in Table 2, line 3, and photos are shown in Figure 2A. Ants having pieces of onion in their foraging area poorly oriented themselves towards a tied nestmate. They failed in reaching this attractive stimulus or went over it. The obtained median value of orientation (69.0 ang. deg.) almost equaled that previously found for such maintained ants (65.0 ang. deg.).[6] After they consumed the homeopathic product since two days, these ants living in presence of onion presented an orientation just not significantly different (so not better) than that they had while not consuming the product (χ^2=5.66, df=2, 0.05 < P < 0.10). Consequently, Ignatia Mk did not decrease the stressing effect of onion, appearing thus, as in the previous experiment, inefficient, or at least far too ineffective. As a control, ants maintained under normal conditions (with no onion odor and no Ignatia Mk) very well orientated themselves towards a tied nestmate. The difference between their value of orientation and that of ants in presence of onion and consuming the drug product was statistically

significant: χ^2=33.28, df=2, P < 0.001. We can thus conclude that Ignatia Mk was unable to decrease the adverse effect of onion on the ants' orientation ability.

Audacity

Results can be found in Table 2, line3, and photos in Figure 2B. With pieces of onion in their foraging area, ants were little inclined in coming onto the presented risky unknown apparatus, and if coming on it, they soon went away. The mean value of ants sighted on the apparatus (1.50) approached that previously found for such maintained ants (1.65).[6] When having in addition the homeopathic product at their disposal, the ants were still reluctant in coming onto the unknown apparatus, and those which came on it shortly stayed there. The obtained results statistically did not differ from those obtained on ants having onion in their area and not consuming Ignatia Mk (N=4, T=+4, -6, P=0.438). Consequently, the latter product did not reduce the adverse effect of onion on the ants' physiology and behavior. When maintained under normal conditions, the ants were far more inclined to come onto the presented unknown apparatus. The difference between the numbers of workers, living under normal conditions or with onion and Ignatia Mk, sighted on this apparatus was significant: N=5, T=15, P=0.031. Therefore, there was really no doubt that ants were in good, usual health, and that Ignatia Mk was unable to counteract the adverse effect of onion.

Tactile perception

Table 2, last lines, gives the results and photos are shown in Figure 2C. Ants living with onion in their foraging area did not walk very slowly and sinuously on the provided rough substrate, thus poorly perceiving its uncomfortable character. The obtained median values of speeds (8.1mm/s, 138 ang.deg./cm) were spectacularly similar to those previously obtained for ants living under the same condition (8.0mm/s, 135ang.deg./cm).[6] While they consumed the homeopathic product, these ants perturbed by onion went on poorly perceiving the uncomfortable character of the substrate: they seldom touched the substrate with their antennae, and walked on it rather quickly and not very sinuously. The obtained numerical results did not statistically differ from those corresponding to ants not consuming Ignatia M K and living with onion in their area: linear speed: χ^2=0, df=1, NS; angular speed: χ^2=1.03, df=2, 0.50 < P < 0.70. It can thus be concluded that the homeopathic product did not allow ants recovering from the onion adverse effect on their sensory perception. While living again under normal conditions, the ants very slowly and sinuously waked on the rough substrate, clearly perceiving its uncomfortable character. The difference between this locomotion and that presented by ants having onion around them and Ignatia M^k in their sugar water was significant: linear speed: χ^2=25.17, df=1, P < 0.001; angular speed: χ^2=40.61, df=1, P < 0.001. It could thus be admitted that the experimented ants were as usual, in good health and that the homeopathic product could not reduce the adverse effect of onion.

Brood caring

Numerical results are given in Table 3, line 1, and photos are shown in Figure 2D. Under onion perturbation, the ants badly re-entered the larvae experimentally removed from the nest. They delayed in finding the larvae located near them; they had difficulty in taking them correctly in their mandibles. Consequently, after the five experimental minutes, there were still 4 not re-entered larvae among the 10 observed. In our previous work on homeopathy, we also found such a number of not re-entered larvae after five minutes.[6] When the homeopathic product was provided to these ants having onion in their foraging area, the same events occurred, and since several larvae had then reached the nymph stage and were thus more difficult to be transported, there were still 7 ones not yet re-entered after the five experimental minutes. It can be concluded that Ignatia Mk did not improve the ants' brood caring behavior impacted by the onion odor. Thereafter, while living in the absence of onion and the homeopathic drug, the ants rapidly re-entered the larvae experimentally removed from the nest. As soon as after 3 ½ minutes, all the 10 observed larvae were re-entered. The difference of the numbers of not re-entered larvae over time between ants maintained under normal conditions or in the presence of onion and Ignatia Mk was significant: N=6, T=21, P=0.016. The experimented ants behaved thus as usually, but were affected by onion odor, and Ignatia Mk did not reduce the adverse effect of onion. This homeopathic product was here inefficient.

Social relationships

Numerical results can be found in Table 3, line 2, and photos seen in Figure 2E. While living with pieces of onion in their foraging area, during the experimental dyadic encounters, the ants did not behave completely peacefully. They did not often stay calmly side by side, and they often opened their mandibles. The variable quantifying their level of aggressiveness equaled 0.82, what was rather high. In our first work on homeopathy, ants having onion in their foraging area had also a rather high such variable, i.e. 0.69.[6] When consuming the homeopathic product, these ants, whose foraging area still contained onion, once more behaved not entirely peacefully. They often opened their mandibles, and seldom stayed calmly side by side. Statistically, there was no difference as for the distributions of the ants' values of aggressive levels, between the ants living in presence of onion or living in presence of onion and consuming Ignatia Mk. Consequently, the latter homeopathic product was inefficient in reducing the stressing, perturbing effect of onion. Thereafter, when maintained under normal conditions, the ants observed during the experimental encounters often stayed near each other, doing nothing or antennal contacts, and sometimes slightly opening their mandibles. The difference between the ants normally maintained or maintained in presence of onion and Ignatia Mk as for their recorded numbers of aggressive levels was highly significant: χ^2=43.29, df=2, P < 0.001. Consequently, the ants were initially not aggressive, they became so in the presence of onion, and Ignitia Mk could not reduce this adverse effect, i.e. it looked like an inefficient drug.

Escaping ability (state of stress and cognition)

Results are reported in Table 3, line 3, and photos are shown in Figure 2F. With onion in their foraging area, the ants poorly succeeded in escaping from the experimental enclosure. They walked erratically all around the enclosure, not calmly along its rim, and when walking there, they often moved past the exit without using it. Also, if approaching the exit, they had little tendency to go out. This behavior was in agreement with that of ants presented with a risky unknown apparatus (see the subsection relative to audacity). After the twelve experimental minutes, only 4 ants among 12 could escape. In our previous work on homeopathy, in presence of onion, only 7 ants among 12 could escape.[6] Onion increased thus the ants' state of stress, and probably somewhat decreased their cognition, a hypothesis examined in the following subsection. When these ants living in the presence of onion additionally consumed the homeopathic product, they were again poorly able to escape from the enclosure, stressing, walking erratically in the enclosure, and generally not going out when walking near the exit. After the twelve experimental minutes, only 3 ants among 12 could escape. There was no statistical difference between this result and that obtained for ants living in presence of onion and not consuming Ignatia Mk (N=2, NS). Consequently, the

latter homeopathic product did not reduce the impact of onion on the ants' state of stress and cognition. When ants were maintained under normal conditions (i.e. without onion and Ignatia Mk), they were more able to go out of the enclosure. They did not stress a lot of. One ant among 12 could escape as soon as after one minute, and 11 among 12 ants were escaped after the 12 experimental minutes. The difference between the numbers of escaped ants over time between the ants under normal maintenance and those living with onion and Ignatia Mk was significant: N=5, T=15, P=0.031. It could thus be concluded that the experimented ants behaved normally, that onion induced stress and somewhat impacted cognitive abilities, and that the homeopathic product could not counteract the adverse effect of onion, i.e. it was inefficient.

Cognition

Table 3 gives the results in its last line, and Figure 3A shows photos. While having onion in their foraging area, the ants were little inclined in entering the twists and turns path, and even if entering it, they often went back on their way, and very seldom succeeded in fully crossing it. In fact, after the twelve experimental minutes, only 2 ants among 30 could cross this difficult path and 14 ones were still in front of the path. This agreed with the ants' behavior in front of an unknown risky apparatus (see the subsection relative to audacity). When performing this experiment in the course of our first work on homeopathy, we found that, after the twelve experimental minutes, 2 ants had crossed the twists and turns path and 15 ones were still in front of it.[6] Our present and previous works are thus in agreement with each other according to this examined trait, i.e. the ants' cognition. When having the homeopathic product at their disposal and still pieces of onion in their foraging area, the ants were again poorly able to cross the twists and turns path in the course of the twelve experimental minutes. The difference of behavior between the ants living under the two kinds of maintenance was at the limit of significance for their numbers still in front of the difficult path (N=4, T=10, P=0.063; with the ants consuming Ignatia Mk having the lower ability in crossing the path), and was not significant as for their numbers having reached the zone beyond the path (N=2, NS). Consequently, the homeopathic examined product was inefficient in reducing the impact of onion odor on the ants' cognition (and tendency to explore as already shown through the experiment relative to the ants' audacity). Under normal condition, the ants were statistically more able to cross the twists and turns path; after the twelve experimental minutes, 9 ants were beyond that difficult path. The difference between the ants under normal maintenance and those living in presence of onion and Ignatia Mk as for the numbers of workers being in front and beyond the twists and turns path was statistically significant: in front: N=6, T=21, P=0.016; beyond: N=5, T=15, P=0.031. It could thus be concluded that the ants had their cognition intact, that onion impacted it, and that Ignatia Mk was inefficient in reducing this plant's adverse effect.

Conditioning acquisition, memory

Table 4 gives the results, and Figure 3B shows photos. Ants living with pieces of onion in their foraging area acquired a low-level operant conditioning, if any. Their behavior in the Y-maze resembled that in front of a risky apparatus and of a twists and turns path: they were rather reluctant in progressing in the maze and in going under the hollow green cube, so in giving the correct response. After seventy two training hours, these ants reached a conditioning score of only 65%. After the green cube removal, the ants lost their conditioning in about 50 hours, and this also occurred in our first work on homeopathy, under the same circumstances.[6] When provided with a solution of the homeopathic product, these ants having onion in their foraging area again reached a conditioning score of 65%. There was no statistical difference between their conditioning acquisition and that they presented while having no Ignitia Mk at their disposal. Therefore, the latter homeopathic product did not improve the ants' leaning impacted by the onion odor. After the cue removal, the ants consuming the homeopathic drug while living in presence of onion lost their conditioning nearly as those not consuming this homeopathic drug. Statistically, there was no difference between the responses of the ants maintained under one or the other kinds of conditions (N=2, NS). Consequently, Ignatia Mk did not ameliorate the ants' memory impacted by the permanent odor of onion. Under normal condition (i.e. without onion and Ignatia Mk), the ants soon acquired conditioning; they reached a score of 80% after 48 training hours and a score of 90% after such 72 hours. The difference, as for the six successively assessed scores, between the ants under normal maintenance and those having onion in their area and Ignatia Mk at their disposal was statistically significant: N=6, T=21, P=0.016. After the blue cube removal, the ants under normal maintenance kept their conditioning over 72 hours. The difference between their six successive conditioning scores and those presented by ants living with onion and Ignatia Mk was significant: N=6, T=21, P=0.016. It could thus be concluded that the ants initially had an intact memory, that their memory was affected by onion odor, and that Ignatia Mk was inefficient in conteracting this adverse effect.

Conclusion

Physiologically and behaviorally, Ignatia amara Mk appeared to induce no improvement of the ants' behavior, whatever the examined trait is, i.e. their food intake, activity, audacity, tactile perception, social relationships, state of stress, cognition, learning and memory. We therefore can conclude that the significant differences we observed in our previous study on ants experiencing a stress caused by onion and maintained either under a normal diet or under a diet including Ignata amara 5CH were not caused by the nature of the globules used for delivering the homeopathic drug. As we set up in place the same experimental protocol than that used in the previous study, it is unlikely that the current result could have been affected by some statistical issues. So, it seems that there is actually an effect of the diluted solution of Ignata amara 5 CH on ants' physiology and behavior. We are still unable to propose any strong hypothesis to explain the cause of this observed effect, as a 5 CH homeopathic drug represent a 10^{-10} dilution of the initial concentration of an Ignata amara solution, but it is likely that, even at very small concentration, the active substance (i.e. strychnine) produces some effect on the ants' physiology.

As regards the use of homeopathic drug in the field of human health, and considering the fact that at dilution below 12CH there are, theoretically, no more active molecule in the diluted solution, we can recommend to base the treatment on the placebo effect. In other words, if the patient's health impairments are caused by psychological disorders, anxiousness as for their work, life conditions, or family members, it could be a good strategy to base their treatment on homeopathic drugs (Ignata amara for instance, being any CH or even MK dosages). This placebo-based treatment could be accompanied by psychological follow-up or by other stress-reducing activities. Such persons could have freely Ignatia amara Mk at their disposal (all the more since we detected no adverse effect for this product in our study). Nevertheless, such persons caring of themselves with Ignatia Mk (and at a lower extent with Ignatia CH) must be carefully monitored as for their different physiological health problems (anxiousness, stress, food consumption, pains, bad sleeping, etc …), since Ignatia amara Mk appeared to be inefficient on ants as model organisms for treating

such impairments. Indeed, alongside to psychological troubles, these patients may suffer from some physiological disorders and, very likely, these symptoms will not be cured thanks to homeopathic drugs, at least if using large dilution rates (fewer than 10CH or 10K). Therefore, these patients must be tried to accept adequate true medicines if serious physiological or psychological symptoms do not disappear. Surprisingly, Ignata amara 5CH seems to be an effective drug to release some mild stress-related symptoms (i.e. not those resulting from true severe physiological or psychological illness), as shown in our previous study. Nevertheless, because the mechanism of action of this drug remains unknown to us, Ignatia amara 5CH cannot be recommended as an alternative to a drug with a known mechanism of action for solving severe symptoms which may endanger the patient's health.

Acknowledgments

None.

Conflict of Interest

We affirm having no conflict of interest as for the use of any homeopathic product. Marie-Claire Cammaerts works on ants, on their behavior and cognition, and uses them as models for examining several subjects. David Cammaerts works on natural water quality and related subjects, and teaches and makes research on these subjects.

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