Research Article

Adverse effects of the dietary supplement berberine studied on ants as models

Abstract

An extract of *Berberis* plants is nowadays used as a dietary supplement as well as for treating several medical symptoms, e.g., mostly for regulating glycemia, lipid metabolism and central nervous system disorders. Few or no information has so far been reported about side effects of berberine on behavioral and physiological-linked traits. We thus investigated on the potential adverse effects of the alkaloid berberine by using the ant *Myrmica sabuleti* as a model. We found that berberine decreased the ant's food intake, activity, locomotion, and sensory perception, but did not impact their social interactions, cognition, learning and memorization. The ants did not adapt themselves to the side effect of berberine on their locomotion. They did not develop strong dependence on berberine consumption. After weaning, the behavioral effect of a diet with berberine linearly decreased until it vanished in 15 – 18 hours. Researches to assess the effect of berberine on various associated behavioral and physiological traits in mammals and in humans are still needed in order to draw a complete safety profile of this substance.

Keywords: ant model, behavioral impairments, berberine, cognition, sensory perception

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Abbreviations: ang.deg., angular degree; ang. deg./cm, angular degree per centimeter; CNS, central nervous system FDA, U.S. Food and Drug Administration; h, hour; HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; min, minute; ml, milliliter; mm/s, millimeter per second; N°, n°, number; s, ", second

Introduction

Although plants containing berberine are used since already 3000 years in the Ayurvedic and Chinese pharmacopeias,[1] this substance was quite recently available as an over-the-counter dietary supplement. With recognized or alleged therapeutic properties, it is used among other beneficial effects, to regulate glycemia and lipid metabolism[2] as well as for treating CNS disorders such as depression, anxiety, cerebral ischemia, schizophrenia, Alzheimers' and Parkinsons' diseases[3–6] and several other health dysfunctions.[7] Few side effects at common doses are reported in the literature, and those that may lead to neurological or behavioral disorders are rarely if ever reported. We therefore intended to fill this gap using the behavior of an ant as a biological model. Before explaining our work, we shall outline what is known about the medical properties of berberine and its safety. Then, we shall explain why an ant was used as a model and which species we used, what we know about its behavior and which of its ethological and physiological traits were examined in order to assess the safety of berberine as a dietary supplement.

Berberine as a medication

Berberine is a benzylisoquinoline alkaloid (Figure 1) present in several plants, among others in barberry (*Berberis vulgaris*) and tree turmeric (*B. aristata*) shrubs.[1] Mostly used as a dietary supplement it is reported to have a broad spectrum of pharmacological effects, not always supported by strong evidence. Nevertheless, its medicinal properties include hypoglycemic, hypotensional, hypolipidic, anti-oxidant, anti-inflammatory, immunomodulating and neurological effects.[1] Berberine is mainly distributed in the body tissues by the liver and metabolized in it, where it regulates glucose and lipid disorders, and in the gut where its regulation of the microbiota imbalance also produces this effect.[2,8,9] The modes of action of berberine are still under investigation. We here relate some significant findings about its medical effects.

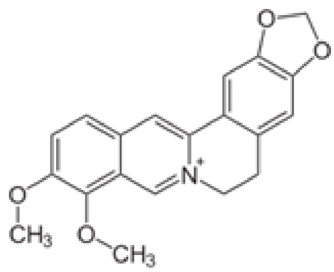

Figure 1 Chemical structure of berberine.

The effect of a root extract of *B. aristata* on antioxidant and carbohydrate metabolism regulating liver enzymes was assessed during 21 days on alloxan-induced diabetic rats and found to significantly play a role in glucose homeostasis by lowering the blood glucose without a hypoglycemic effect.[10] Another study of the same kind also showed an antihyperglycemic effect, with a significant reduction of glucose level at the 15th day of treatment compared to a control group of rats. Moreover, the levels of cholesterol and triglycerides significantly decreased in the diabetic group treated with the *B. aristata* extract while its level of high-density lipoprotein cholesterol (HDL-c) increased.[11] The in vitro hypoglycemic properties of plant extracts of *B. aristata* and *Tamarindus indica* were also evaluated, thanks to their effects on glucose adsorption capacity, glucose diffusion, amylolysis kinetics and glucose transport across yeast cells. Each of these plant extracts adsorbed glucose, promoted glucose uptake by the yeast cells and significantly inhibited the movement of glucose into an external solution across a dialysis membrane as compared to control. At all concentrations, the hypoglycemic activity of the *B. aristata* extract was significantly higher than that of the *T. indica* extract.[12]

The effect of an extract of barberry (*B. vulgaris*) fruit was investigated during 3 months in a double-blind randomized clinical study, on 42 type 2 diabetic patients divided into two groups of 21, one treated with the extract and the other treated with a placebo. After three months, there was a significant decrease in e.g., serum glucose, LDL-c and insulin in the patients treated with the extract compared to those that received only placebo.[13] In another clinical randomized, double-blind and placebo-controlled study on a small sample of 13 patients with type 2 diabetes mellitus the effect of a *Berberis sp.* fruit extract to achieve glycemic control showed a significant reduction in serum glucose and glycated hemoglobin level in the treated patients.[14] Thus, a plant extract containing berberine could improve the natural glucose catabolism. A meta-analysis concerning seven Iranian studies and 452 participants examined the effect of extracts or juice of barberry on glycemic markers. It showed that barberry lowered the insulin level ($P = 0.04$), but that, contrary to other clinical trials, no significant changes occurred for fasting blood sugar (FBS), glycosylated hemoglobin (HbA1c) and homeostatic model assessment for insulin resistance (HOMA-IR).[15] In a double-blind clinical trial made while 80 newly diagnosed type 2 diabetes mellitus patients continued their current hypoglycemic medication, a group of 40 patients took a root extract of *B. vulgaris*, and another group of 40, a placebo. After six weeks, the amounts of fasting blood sugar and fasting insulin as well as those of total cholesterol and LDL-c were significantly lower in the former group compared to the latter group and without important side effects.[16] Patients with low cardiovascular risks treated with *B. aristata* and *Silybum marianum* extracts were found, in a double-blind study involving 105 individuals, to present a mildly and safely reduction of their cholesterol, triglycerides and LDL-c levels as well as an increased HDL-c, as long as they were treated with a plot berberine.[17]

A pilot study determined during three months the efficacy of the berberine molecule itself compared to metformin monotherapy in the treatment of 36 diabetes mellitus patients. Similar to metformin treatment, a significant decrease in blood glucose, triglycerides and cholesterol was observed under berberine monotherapy. In another group of 48 patients with poorly controlled diabetes mellitus, blood glucose, total cholesterol and LDL-c were significantly reduced when berberine was combined with their still inadequate treatment.[18] A meta-analysis of 11 randomized controlled trials on the effect of the berberine molecule on blood lipids concluded that this substance reduced significantly total cholesterol, triglycerides and LDL-c while it increased HDL-c, but also that it should be further evaluated in a larger population.[19]

Besides somatic effects, berberine is reported to have beneficial effects on various neurodegenerative and neuropsychiatric disorders such as having a stunt action in Alzheimer disease progression, and protective effects on forebrain ischemia, mental depression, schizophrenia and anxiety.[3–6] One of the molecular actions of berberine is to inhibit the monoamine oxidase enzyme what enhances the availability of biogenic amines such as norepinephrine and its precursor, dopamine, a CNS neurotransmitter which affects several behavioral traits. Indeed, at doses of 5 to 10 mg/kg/day, berberine significantly increased the dopamine and norepinephrine levels in the brain of mice and had a positive effect on their level of locomotor activity.[20] Administrated at 50 mg/kg to rats developing depression-like and anxiety-like behavior under morphine discontinuation, berberine decreased their time of immobility and restored their climbing and exploratory behavior.[21] However, another study provided opposite results, as rats treated with 5 to 30 mg/kg/day berberine experienced a significantly decrease in dopamine levels while a cell culture treated with berberine at 30µM resulted in a significant increase of apoptotic cells.[22] Although some of the results were contradictory, they do not deny that berberine can act on the brain's dopamine levels. In Drosophila flies, the level of dopamine has been shown to regulate vitality, feeding appetite and certain aspects of their locomotor behavior,[23] while in honeybees it enhanced appetitive response, olfactory learning and memory.[24] It is also known that, in human individuals, the highly polymorphic Drd4 gene, which influences the postsynaptic action of dopamine, is associated to the level of novelty-seeking and impulsivity[25] and to neurologic and psychiatric disorders.[26] In a passerine bird, *Parus major*, this gene polymorphism was found to be associated with the individual variation in curiosity.[27]

Safety of berberine

Clinical trials contain some sparse information on side effects of berberine. Although high doses of berberine can result in severe adverse effects such as a lowered blood pressure, dyspnea, flu-like symptoms, and cardiac damage,[28] only mild to moderate gastrointestinal discomfort was reported at doses used in clinical treatments against hyperglycemia or hyperlipidemia albeit in one study, some 35% of 66 patients under berberine medication suffered from it.[18] Reported discomfort events were diarrhea,[18,19] constipation,[18,19,29] flatulence,[18,19] heartburn[16] and 'abdominal pain'.[18,30] Other somatic impairments such as allergic skin reaction and arrhythmia (Ding 2002 in[31]) were also reported. Berberine neurologic side effects were however not reported, if we except that this substance is suspected to cause, by an elevation of free bilirubin in the blood, jaundice and kernicterus in neonates with glucose-6-phosphate dehydrogenase deficiency.[31] On the basis of the scientific literature, berberine appears thus reasonably safe. However, it is worth noting that a review of the pharmacokinetic and pharmacodynamic features of the chemical substance berberine, including its safety profile, concluded that studies to detect rare adverse effects of berberine need to be initiated to draw a complete safety profile of this product and to strengthen its applicability.[1] A– literature overview of endocrinological and therapeutical effects of an extract of barberry on various body organs also concluded that more targeted and intensive researches are needed.[7] Being a dietary supplement berberine cannot be approved by the FDA. However, there are concerns about a toxic mode of action of this stuff delivered over-the-counter, so that the European Commission presently requested the European Food Safety Authority (EFSA)[32] to assess the available information on the safety of berberine in order to provide an independent scientific advice. On the whole, few or no information has so far been reported about potential effects of berberine on behavioral and physiological-linked traits. The aim of the present study is to fill this gap somewhat, by using ants as a biological model.

Why using ants

Basic biological functions are identical in every animal species including humans (genetics, nervous system, muscle contraction, sensory perception, conditioning acquisition). This is why the effect of substances on humans is firstly studied on vertebrates and invertebrates.[33] Invertebrates are often preferred because they are small, easily maintained at low cost in a laboratory, and have a short reproductive cycle.[34] Insects such as Hymenoptera (e.g., bees) are used,[35] and ants can thus also be used. They can be the more so since several colonies containing hundreds of ants can be kept during months and even years, easily and at very low cost.

Which species we used and what we know on it

The present experimental work was made on the ant *Myrmica sabuleti* Meinert, 1861, the biology of which is rather well known. Indeed, its visual perception, navigation, and recruitment strategy,[36] the ontogenesis of some of its skills,[37] its self-recognition skill,[38] its reaction to distance and size values,[39] its perception in line with Weber's law,[40] as well as its numerosity abilities and related topics[41–44] were already investigated. Knowledge of these characteristics makes this ant a suitable biological model.

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Which traits we examined

We aimed to examine the impact of berberine on the food intake, activity, locomotion, orientation, audacity, tactile perception, social relationships, stress, cognition, learning and memory of *M. sabuleti* forager workers as well as their adaptation to side effects of berberine on their locomotion, dependence on berberine consumption, and decrease of the effect of this dietary supplement after its use was stopped.

Materials and methods

The following subsections concern the here used materials and methods which were similar to those employed to examine the effects of 67 other substances and situations used, consumed or lived by humans. These methods have been detailed in many previous publications together with photos of the different used experimental protocols, e.g., in[45–48] We thus related them here as briefly as possible, however without being enable to avoid some self-plagiarism.

Collection and maintenance of ants

Three ant colonies of *Myrmica sabuleti* Meinert, 1861 (two used for the experiments and one just for a particular control) were collected in 2022 in an abandoned quarry located in the Aise valley (Ardenne, Belgium). The colonies contained about 500 to 1,000 workers, 1 to 3 queens and brood. They were maintained in 1 to 3 glass tubes half filled with water, a cotton plug separating the water compartment from that devoted to the ants' nesting. The nest tubes of each three colonies were set in a tray, the borders of which having been slightly covered with talc. The trays served as foraging areas, in which food was delivered, i.e., *Tenebrio molitor* larvae three times per week and sugar water permanently delivered in small cotton-plugged tubes. The lighting varied from 330 to 110 lux, the humidity equaled 80%, and the temperature 20°C.

Solution of berberine given to the ants

Capsules containing 250 mg of berberine were bought from the pharmacist Wera (1170, Bruxelles, Belgium). The manufacturer of this dietary supplement AS.587/225 is DeBa Pharma (Dumolinlaan, 13, 8500 Kortrijk, Belgium). Humans using this dietary supplement are advised to consume 1 or 2 of these capsules per day. Generally, humans consume about one liter of water per day. So, while living under a diet with berberine, they ingest maximally 2 capsules of the product and drink about one liter of water. Due to the physiology of their excretory apparatus and the cuticular nature of their exoskeleton, the insects consume about ten less water than mammals. Therefore, to maintain the ants under a diet with berberine similar in proportion to that of humans consuming 2 capsules of this product, they must be provided with a solution of two capsules of berberine dissolved into 100 ml of their usual drink, which is a solution of 15% of saccharose in water. Such a sugared water solution of berberine was thus furnished to the ants in small cotton-plugged tubes instead of their usual sugar water cotton-plugged tubes. The plugs of these tubes were refreshed every 2-3 days, and their content was renewed every seven days. We checked each day if ants drunk that solution, and they did. We firstly performed the control experiments on the three used colonies maintained under their usual sugar water normal diet. Then, we replaced the tubes of the two experimental colonies by tubes containing the sugared solution of berberine, and began the test experiments 12 hours later.

Food intake, activity

First for ants under normal maintenance, thereafter for those having berberine in their sugar water, for each of the two colonies used for the experiments, we counted four times per day during six days (n = 4 x 2 x 6 = 48 counts), the workers being on the meat food, those being in front of the sugar water tube, and those being active at any place in their environment (foraging area, nest entrance, inside the nest). For each kind of count and each diet, the daily means of these counts were established (Table 1, lines I to VI). The six daily means for each trait obtained for ants consuming berberine were compared to those obtained for ants under normal diet by using the non-parametric test of Wilcoxon.[49] The means of the six daily means were also established (Table 1, line I-VI).

Table 1 Impact of berberine on the ants' food intake and activity. The table gives the daily mean numbers of ants sighted on their meat and their sugar water, and being active (lines I to VI), as well as the mean of these six daily means (line I-VI). Berberine affected these biological traits

Days	Under normal diet			Under a diet with berberine		
	meat	sugar water	activity	meat	sugar water	activity
I	1.00	1.50	12.00	0.25	0.63	5.75
II	1.00	1.25	9.13	0.25	0.63	4.50
III	1.25	1.50	8.88	0.25	0.80	3.75
IV	1.75	1.00	8.00	0.25	0.88	5.12
V	1.63	1.34	7.50	0.25	0.75	5.00
VI	1.25	1.34	7.50	0.25	0.63	4.63
I-VI	1.30	1.32	8.84	0.25	0.72	4.79

Speed and orientation

The ants' linear and angular speeds were quantified while they walked in their foraging area, without stimulating them. Their orientation was quantified while they were stimulated by a nestmate tied to a piece of paper. This tied nestmate emitted the attractive alarm pheromone of its mandible glands, what induced the surrounding ants to approach it. To quantify the ants' speeds and their orientation, 40 of their trajectories were recorded and analyzed using appropriate software.[50] Linear speed (mm/s) is the length of a trajectory divided by the time spent to travel it. Angular speed (ang.deg./cm) is the sum of the angles made by successive adjacent segments, divided by the length of the trajectory. Orientation (ang. deg.) to a location is 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 inferior to 90° signifies that the observed individual approaches the location. A value superior to 90° signifies that it avoids the location. For the three variables, the median and quartiles of the 40 recorded trajectories were calculated (Table 2, lines 1,2,3). The distributions of the 40 values obtained for ants consuming berberine were compared to those obtained for ants living under normal condition by using the non-parametric χ^2 test.[49]

Table 2 Impact of berberine on the ants' locomotion, orientation ability, audacity and tactile perception. The table gives the median (and quartiles) or the mean [and extremes] of the recorded data. Berberine affected these five biological traits

Traits	Under normal diet	Under diet with berberine
Linear speed (mm/s)	9.4 (8.3–10.8)	4.7 (4.1–5.5)
Angular speed (ang.deg./cm)	120 (104–140)	225 (198– 247)
Orientation (ang.deg.)	30.0 (22.6–38.8)	82.5 (68.9– 98.9)
Audacity (n° of ants)	2.50 [1–4]	0.53 [0–1]
Tactile perception		
Linear speed	2.4 (2.1–2.5)	4.8 (4.3–5.3)
Angular speed	376 (359–448)	213 (191–235)

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Audacity

This trait was evaluated by the number of ants coming onto an unknown apparatus made of Steinbach® white paper, i.e., a cylinder (height = 4 cm; diameter = 1.5 cm) vertically tied to a squared platform (9 cm^2) and deposited in the ants' foraging area. The ants coming on this apparatus were counted 20 times over 10 minutes (n =20 x 2 = 40) and the mean and extremes of these counts were established (Table 2, line 4). The numbers obtained for the two colonies were correspondingly added, the twenty obtained sums were chronologically added two by two and the ten numbers finally obtained for ants consuming berberine were compared to the ten numbers obtained for ants under normal diet, by using the non-parametric Wilcoxon test.[49]

Tactile perception

This trait was evaluated through the walking of ants on a rough substrate. When they perceive the rough character of a substrate, the ants walk on it slowly, sinuously, with difficulty and often touch the substrate with their antennae. If they do not perceive the roughness of a substrate (e.g., if their perception is affected by some factor), the ants walk on it less sinuously, rather rapidly, and seldom touch it with their antennae. A strip of emery paper n°280, 3 cm wide x 11 cm long (2 + 7 + 2 cm), was inserted flat across the middle of a tray measuring 7 cm wide (as well as 15 cm long and 4.5 cm high). The extra 2 cm on each side of the flat 7 cm strip of emery paper were each folded vertically against the side of the tray, holding the flat section in position and deterring ants from crossing the rough surface by climbing up the sides of the tray. To conduct an experiment, 25 ants of each experimented colony were transferred inside the tray at one side of the emery paper and their linear and angular speeds were quantified while they walked on or crossed the rough substrate. The distributions of these values were compared to those previously obtained for ants consuming berberine and moving in their foraging area, by using the non-parametric χ^2 test,[49] this allowing to evaluate the effect of the dietary supplement on the ants' tactile perception. In addition, for each kind of diet, the 40 values of linear and angular speeds of ants moving on the rough substrate were characterized by their median and quartiles (Table 2, lines 5,6).

Brood caring

Some larvae were taken out of the nest and set near the entrance. Only five of them per colony were followed because they had to be simultaneously observed. The ants' behavior towards them was observed during 5 minutes and the number of larvae not re-entered was counted after 30 seconds, 1,2,3,4, and 5 minutes (Table 3, line 1).The six numbers of not re-entered larvae chronologically recorded over time for the two colonies were correspondingly added, and the six sums so obtained for ants consuming berberine were compared to the six sums obtained for ants normally maintained, by using the non-parametric test of Wilcoxon.[49] The experiment was not repeated because removing brood from the nest largely perturbated the colony.

Table 3 Impact of berberine on the ants' brood care, social relationships, stress and cognition. The table gives the numbers of larvae or ants over time, or of the levels of aggressiveness and the variable a (see explanation in the text). Berberine did not impact these four traits

Trait	Under normal diet						Under a diet with berberine					
N° of not re-entered larvae over 5min	30"	1'	2'	3'	4'	5'	30"	1'	2'	3'	4'	5'
	10	8	6	4	0	0	11	10	8	6	4	1
N° of presented levels of	0	1	2	3	4	'a'	0	1	2	3	4	'a'
aggressiveness; variable 'a'	58	44	6	0	0	0.06	45	34	7	0	0	0.09
N° of escaped ants over 12 min	2'	4'	6'	8'	10'	12'	2'	4'	6'	8'	10'	12'
	3	5	8	10	12	12	3	4	6	10	12	12
N° of ants	2'	4'	6'	8'	10'	12'	2'	4'	6'	8'	10'	12'
in front (f) and beyond (b)	f: 23	21	18	15	14	11	f: 26	20	20	16	14	12
a twists and turns path over 12 min	b: 0	2	4	6	8	10	b: 0	0	2	2	4	8

Social relationships

Ants pertaining to a same colony do not aggress each other, but environmental factors may impact this peaceful behavior. To know if berberine does so, for each colony, five dyadic encounters were conducted between nestmates (for each kind of diet: n = 5 x 2 observed encounters). The encounters took place in a polyacetate talked cup (diameter = 2 cm, height = 1.6 cm), and one ant of each pair was carefully observed during 5 minutes. Its behavior was defined thanks to the numbers of times it did nothing (level 0 of aggressiveness), touched the opponent ant with its antennae (level 1), opened its mandibles (level 2), gripped and/or pulled the opponent (level 3), and tried to sting or stung it (level 4) (Table 3, line 2). For each level of aggressiveness, the numbers obtained for the 10 observed ants were correspondingly added, and the distribution of values obtained for ants consuming berberine was compared to that obtained for ants normally maintained using the non-parametric χ^2 test.[49] Also, for each diet, a variable 'a', which equaled the number of aggressiveness levels 2 + 3 + 4 divided by the number of aggressiveness levels 0 + 1, was established (Table 3, line 2).

Stress and cognition

To be able to go out of an enclosure, an individual must stay calm, not stress, looks for an exit, and has his cognitive ability intact. For estimating the impact of berberine on the ants' state of stress and cognition, six ants of each colony were enclosed under a reversed talked cup (made of polyacetate; height = 8 cm, bottom diameter = 7 cm, ceiling diameter = 5 cm) deposited in their foraging area. A notch (3 mm height, 2 mm width) had been made in the rim of the bottom of this cup for giving to the ants the possibility of escaping. For each colony, the escaping after 2, 4, 6, 8, 10 and 12 minutes were counted. The numbers obtained for the two colonies were correspondingly added (Table 3, line 3), and the six sums obtained for ants consuming berberine were compared to the six ones obtained for ants normally maintained, by using the non-parametric Wilcoxon test.[49]

Cognition

The ants' cognition was evaluated through their ability to cross a twists and turns path. For each colony, two pieces of duly folded Steinbach ® paper (4.5 cm x 12 cm) were inserted in a tray (15 cm x 7 cm x 4.5 cm) in order to create a twists and turns path between a first 2 cm long zone in front of this path and a second 8 cm long zone beyond the part containing the difficult path. To conduct an experiment on a colony, 15 ants were transferred into the first zone, and the ants still there as well as those having reached the second zone, were counted after 2, 4, 6, 8, 10 and 12 minutes. The numbers chronologically obtained for the two colonies were correspondingly added (Table 3, line 4), and for each zone, the summed numbers obtained for ants consuming berberine were compared to those obtained for ants normally maintained, by using the non-parametric Wilcoxon test.[49]

Conditioning acquisition, memory

At a recorded time, a blue hollow cube (constructed in Canson® paper) was deposited above the entrance of the sugar water tube containing berberine near which a piece of mealworm was placed. The ants underwent so operant conditioning. A control experiment on ants kept under normal diet was previously made on a third colony, similar to the two used for the experiments, and used only for this control of conditioning, because once an individual has been conditioned to a stimulus, it keeps this conditioning during a rather long time, and after having lost it, it acquires it again more rapidly than usual. It can thus no longer be used for assessing its conditioning. One and two days after having deposited the cube above the tube containing the sugared water solution with berberine, then one and two days after having removed the cube (the removal was made at the end of the second of these four days), the ants were tested in a Y-maze made of strong white paper, set in another tray, and randomly provided with a blue hollow cube in its left or right branch. To make a test on a colony, 10 ants were one by one transferred in the entrance branch of the maze, and their choice of one or the other branch of the maze was recorded. Choosing the branch containing the cube was giving the correct response. Once it was in one of the branches of the Y-maze, the tested ant was temporarily transferred in a separate cup for avoiding testing twice the same individual, and after that the 10 ants of a colony have been tested, they were all returned into their foraging area. For each of the four performed tests, the responses obtained for the two colonies were correspondingly added, and the overall proportion of correct responses established (Table 4).

Table 4 Impact of berberine on the ants' conditioning acquisition and memory. The table gives the control conditioning scores (%), and the number (n°) of experimented ants giving the correct and the wrong responses as well as their conditioning scores over time (%). Berberine did not affect these two ethological and physiological traits

Elapsed time (hours)	Normal diet (control): conditioning scores (%)	Berberine diet: n° of correct vs wrong responses of colonies A ,	B ;	overall scores (%)
24h	80%	8 vs 2,	8 vs 2;	80%
48h	85%	9 vs 1,	9 vs 1;	90%
Cue removal				
24h	80%	9 vs 1,	9 vs 1;	90%
48h	80%	8 vs 2,	8 vs 2;	80%

Adaptation

An individual adapts himself to a product when he less and less suffers from its side effects over its use. To assess adaptation, we have to rely on a side effect of the product and compare its strength on its initial use with the one after it was used for some time. In the present work, the ants' linear and angular speeds were affected by berberine. These traits were thus assessed after it had been one day of consumption of this dietary supplement in sugar water, and again assessed after having been consumed during seven days. The median and quartiles of the recorded values were established (Table 5 in which the values for normal diet are, moreover, recalled), and the obtained distributions of values were compared by using the non-parametric χ^2 test.[49]

Table 5 Adaptation to a side effect of berberine. Median (and quartiles) of the ants' linear (mm/s) and angular (ang.deg./cm) speeds under three diet situations. The ants did not adapt themselves to the side effect of berberine on their locomotion

Measured trait	Normal diet	+ berberine for 1 day	+ berberine for 7 days
Linear speed	9.4 (8.3 – 10.8)	4.7 (4.1 – 5.5)	4.1 (3.7 – 4.8)
Angular speed	120 (104 – 140)	225 (198 – 247)	203 (160 – 237)

Dependence

An individual acquires dependence on a product when he wants to permanently have the product at his disposal, uses it whatever its adverse effects, and finally no longer lives without using it. The ants' dependence on berberine was examined after the ants had it in their diet during 10 days. For each colony, 15 ants were transferred in an own talked tray (15 cm ×7 cm × 5 cm) inside of which two cotton-plugged tubes (length = 2.5 cm, diam. = 0.5 cm) had been set, one containing sugar water, the other containing a sugared solution of berberine like the one used over the entire experimental work. The tube containing the dietary supplement was located on the right of the tray for one colony and on the left for the other. Then, the ants coming at the entrance of each tube were counted 15 times over 15 minutes, and for each colony and each kind of count, the recorded numbers were added. These two sums obtained for the two colonies were correspondingly added, and the resulting final sums allowed calculating the proportion of ants having gone to each kind of tube (Table 6). The obtained totals were compared with those expected if the ants randomly went towards the two tubes.by using the non-parametric χ^2 goodness-of-fit test.[49]

Decrease of the effect of berberine after its use was stopped

The decrease of the effect of berberine was examined through its effect on the ants' linear speed, after they were under this diet during 12 days. Twelve hours before this study, a fresh sugared solution of berberine was furnished to the ants. From the start of weaning (at t = 0h, when the tubes containing the dietary supplement solution were replaced by ones containing only sugar water), the linear speed of forager ants was assessed every three hours in the same way as it had been assessed after they received the dietary supplement for one as well as for eight days, except that 20 instead of 40 ants' trajectories were recorded and analyzed. This reduction in number of recorded trajectories was made so that after collecting each set of 20 trajectories, the median and quartiles of the speed could be computed in order to estimate when the speed would equal that of the control (i.e., the speed of ants under only normal diet) and allowing so to anticipate the end of the experiment.

For each assessment, the median and quartiles of the recorded values were established (Table 7) and were presented, along with the extreme values, in Figure 2. The successive distributions of linear speed values were compared to the distribution obtained at t = 0 and to the control one using the non-parametric Kruskal-Wallis test for multiple comparisons of independent samples (Table 7). Based on the z values of this test, the resulting P values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure[51] after having choose a false discovery rate (FDR) of 0.05 (Table 7). The Kruskal-Walli's test and the mathematical function describing the obtained increase of the ants' linear speed (i.e., the decrease of the effect of berberine) over time were computed using Statistica® v10 software. The best choice between a first and a second power polynomial regression of the speed increase was established using the procedure described in Zar.[52]

Table 6 Dependence on the use of berberine. Number of ants sighted on a normal diet (sugar water) and on a sugared solution of berberine. The ants appear to have developed a slight, but not significant dependence on this dietary supplement

Sugared solution	Number and proportion of ants' visits to each solution			
	colony A	colony B	total number	proportion
With berberine	13	24	37	62%
Without berberine	8	15	23	38%

Table 7 Decrease of the effect of berberine after its consumption was stopped. This decrease was studied after the ants consumed berberine during 12 days. The table gives the median (and quartiles) of the linear speed (mm/s) presented by ants after they stopped consuming berberine. Briefly, the effect of this product became different from its initial one 3 to 4 hours after weaning and similar to the control situation at about 15 hours after weaning. The decrease was linear. These results are illustrated in Figure 2 and detailed in the text

Time since weaning (hours)	Linear speed (mm/s): median (quartiles)	Kruskal-Wallis statistical results with Benjamini-Hochberg adjustments (P)	
		versus t = 0	versus control
0h	2.7 (2.4 – 3.2)	--	0.0002
3h	4.2 (3.8 – 4.7)	0.0587	0.0002
6h	5.1 (4.3 – 5.5)	0.0064	0.0002
9h	5.5 (4.9 – 6.1)	0.0007	0.0002
12h	6.3 (5.6 – 7.9)	0.0002	0.0003
15h	7.9 (6.7 – 8.6)	0.0002	0.0531
18h	9.2 (8.0 – 11.1)	0.0002	0.4868
control	9.4 (8.3 – 10.8)	0.0002	--

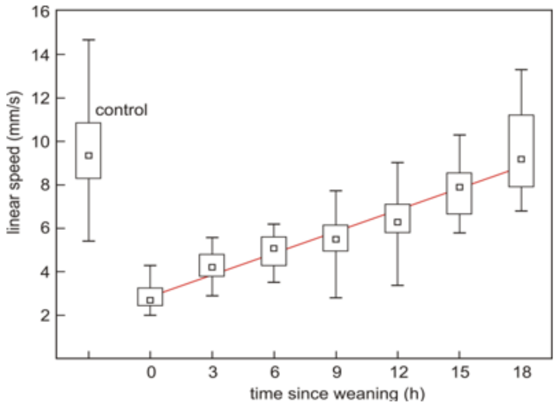

Figure 2 Decrease of the effect of berberine after weaning. The figure shows the medians, quartiles and extremes as well as, in red, the linear relation between the median values. The decrease was studied using the effect of berberine on the ants' linear speed, which varied from minimum 2.0 mm/s at the start of weaning to maximum 13.3 mm/s 18 hours later (median values: 2.7 to 9.2 mm/s), according to a linear function.

Results

Food intake, activity

The meat and sugar water intake as well as the ants' activity were largely negatively impacted by berberine (Table 1). This was obvious for the observers, and statistically significant (N =6, T = -21, P = 0.016 for each trait).

Linear and angular speeds

Berberine affected these two physiological traits (Table 2, lines 1 and 2). Under such a dietary supplement, the ants walked more slowly and more sinuously than while living under normal diet. This was obvious to observers and statistically very significant (linear speed: χ^2=11.43, df =1, P < 0.001; angular speed: χ^2 =24.73, df =1, P < 0.001).

Orientation

Berberine consumption impacted this ethological and physiological trait (Table 2, line 3). On normal diet, the ants oriented themselves very well towards a tied nestmate. On a diet containing berberine, the ants no longer orientated themselves. The difference between the ants under one and the other kinds of diet was statistically very significant (χ^2 = 59.98, df =1, P < 0.001).

Audacity

Berberine affected this biological trait (Table 2, line 4). While ants under normal diet had some valuable tendency to come onto the unknown apparatus, those consuming the dietary supplement were far less inclined to do so. This was obvious to observers and statistically significant (N = 10, T = 5, P = 0.001).

Tactile perception

This physiological trait was affected by berberine consumption (Table 2, lines 5 and 6). Under normal diet, the ants perceived well the rough character of the provided subtract and walked on it slower as well as more sinuously than on their foraging area, the difference being statistically significant (Table 2, lines 1 and 2 compared to lines

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5 and 6: linear speed $\chi^2 = 49.46$, df =1, $P < 0.001$; angular speed $\chi^2 = 59.64$, df =1, $P < 0.001$). On a diet with berberine, the ants did not perceive the uncomfortable character of the rough substrate and walked on it similarly as in their foraging area, i.e., the difference being not significant (Table 2, idem: linear speed $\chi^2 = 1.14$, df =1, $0.20 < P < 0.30$; angular speed $\chi^2 = 0.182$, df =1, $0.50 < P < 0.70$).

Brood caring

Berberine somewhat impacted this ethological trait, but seemingly because these ants had difficulties for finding, then holding the larvae and transporting them inside the nest. Nevertheless, the difference between ants under normal diet and berberine diet as for the number of larvae re-entered over 5 minutes appeared statistically significant (N = 6, T = 21, P = 0.016; Table 3, line 1).

Social relationships

Berberine consumption did not affect this ethological trait (Table 3, line 2). Under normal diet as well as under a diet with berberine, the ants never aggressed nestmates during their encounters. They stayed near one another doing nothing, or making antennal contact, and seldom slightly opened their mandibles. The difference between the ants under one and the other kinds of diet as for their numbers of levels of aggressiveness was accordingly not significant ($\chi^2 = 0.039$, df =1, $0.80 < P < 0.90$). Also, the variable 'a' evaluating the ants' aggressiveness equaled 0.06 for ants under normal diet and 0.09 for ants consuming the dietary supplement.

Stress and cognition

This biological trait, assessed through the ants' escaping behavior, was not affected by berberine consumption (Table 3, line 3). The enclosed ants could all escape in the course of the 12 experimental minutes, and their timing was the same (N = 3, NS).

Cognition

Berberine appeared to slightly affect this biological trait (Table 3, line 4). Under normal diet, the ants could rather rapidly progress inside the twists and turns path and reach the area lying beyond it. While consuming a diet with berberine, they did so more slowly, with some hesitation. However, the difference between the ants under one and the other kinds of diet as for their presence in front as well as beyond the twists and turns path was at the limit of significance (in front: N = 5, T =13, P = 0.094; beyond: N = 5, T = 15, P = 0.063). The reason may be a lower speed of locomotion and general activity under berberine (see the 'Food intake' and 'Speeds' subsections of 'Results'). The possibility that it may be due to some decrease of cognitive abilities was invalidated by the following experiment.

Conditioning acquisition, memory

Berberine consumption did not affect this trait (Table 4). Ants consuming this dietary supplement acquired conditioning exactly as those living under normal diet, i.e., they reached identical and even higher conditioning scores. In the same way, the ants consuming berberine remembered the learned stimulus (i.e., the blue cube) as well as the ants under normal diet (Table 4). There was thus no impact on the ants' cognition.

Adaptation

The ants did not adapt themselves to the impact of berberine on their locomotion (Table 5). Indeed, after seven days under this dietary supplement diet, the ants walked at a linear speed even lower than and at an angular speed similar to the speeds they presented after one day under this diet. This was obvious to the observers and confirmed by statistical results (linear speed: $\chi^2 = 5.33$, df =1, $0.02 < P < 0.05$; angular speed: $\chi^2 = 0.46$, df =1, P =0.50).

Dependence

In front of berberine-free sugar water and of sugar water containing this product, the ants went slightly more often to the berberine solution (Table 6). As regards colony A, the ants made 13 visits to the berberine solution and 8 to sugar water without berberine. For colony B, 24 visits were made to the berberine solution and 15 ones to sugar water only. In total, 37 ants' visits occurred to the dietary supplement solution, and 23 ones to the product-free solution. This corresponded to 62% of the choices to the berberine solution, and 38% to sugar water. The numbers of visits made by the ants (37, 23) did not statistically differ from the numbers (30, 30) which would be obtained if they had randomly visited the two kinds of solutions ($\chi^2 = 1.68$, df =1, $0.10 < P < 0.25$). It therefore could be concluded that berberine did not induce a significant dependence, although a slight preference for berberine may not be excluded.

Decrease of the effect of berberine after its use was stopped

Numerical and main statistical results are given in Table 7 and illustrated in Figure 2. Three hours after weaning, the effect of berberine was already lower (P = 0.0587) than its initial one, albeit at the limit of significance. Six hours after weaning, the effect of berberine became clearly statistically different (P = 0.0064) from its initial one. Continuing to decrease over time, 15 hours after weaning it began to be statistically similar (P = 0.0531) to the control and fully vanished (P = 0.4868) in a total of 18 hours. The decrease of the median values of the linear speed along time (h) is best fitted by a linear regression ($R^2 = 0.9739$) whose equation is: median (mm/s) = 2.83 + 0.33*h. Adding a term of second degree was not statistically significant to the regression model. The long period of time needed for the effect of berberine to disappear completely (between 15 and 18 h) explains the absence of strong dependence on its consumption. Nevertheless, as shown in Figure 2, the decrease appeared to have been slightly more rapid between 0 and 3 hours as well as from 12 hours after weaning, which, if verified by a replicate of the experiment, would account for a possible development of some slight dependence on this product consumption.

Discussion

Although the physiological action, the somatic and neurological health benefits and adverse effects of berberine are still investigated in humans. there exists less information on the behavioral and related physiological effects of this dietary supplement. We therefore wanted to fill this gap somewhat by using an ant as a biological model. We found that, at a dose proportionally similar to that usually used by humans, berberine acted on *M. sabuleti* workers by reducing their food intake, general activity, linear speed, audacity, olfactive orientation, and tactile perception. The observed increase in angular speed (i.e., the sinuosity of movement) may simply result from the reduction in linear speed (indeed, as the sinuosity is equal to the sum of angles along the trajectory divided by the length of the trajectory, a shorter trajectory means a greater sinuosity). Care of the larvae by the workers was also somewhat affected, but seemingly because they had difficulties in holding and transporting them.

The effects of berberine on the ants' locomotory performance (linear speed), general activity, level of audacity and brood care and, to a lesser extent, on their moving in a twists and turns path, are consistent with each other in that they show a negative effect on their vitality. The smaller number of ants observed on the food (sweet and meaty) when they were fed with sugar water containing berberine can most probably not be attributed to a distaste for this substance, since in the dependency experiment there were more workers on the berberine-added sugar water than on the sugar water alone. It may simply result from a decrease of the ants' foraging activity while consuming the dietary supplement. The observed negative berberine effect on the ants' olfactory orientation towards a pheromone source and on their tactile perception indicates a negative impact on the central nervous system (CNS) and/or peripheral sensory organs. In ants[53] and in honeybees,[24] some olfactory memory is known to be established during food exchanges between nestmates (trophallaxis). A decrease of this memory may have affected the *M. sabuleti* workers' research for food and explain the reduced numbers of foraging ants at the food sites. All these negative effects on *M. sabuleti* workers could perhaps be attributed to the hypoglycemic action of berberine if its metabolic pathway in ants is the same as in vertebrates. In short, a lower glucose level in the blood could have led to a reduced vitality. On the contrary, no impact was found on other ethological and/or physiological traits depending from the CNS, such as social relationships between nestmates, stress and cognition, ability to learn and memorize. In contrast to the negative behavioral effects of berberine we observed on *M sabuleti* ants, in which this product is thought to increase the amount of dopamine in the brain, mice that experienced increased dopamine levels in the brain after an intraperitoneal injection of berberine, showed an increased locomotor activity.[20] Contrary also to our observations on ant workers fed with sugar water containing berberine (what should have lead to an increased level of dopamine) and which consequently were less present on the food sites and experienced reduced motility although they kept intact their learning ability, mutant Drosophila fruit flies deficient in dopamine biosynthesis were found to lack appetence (hipophagy), to experience locomotor deficits (walking speed and climbing performance) and to be no longer able to learn an aversive odor, although they still possessed an efficient spatial orientation memory.[23] These contradictory behavioral results between ours on *M. sabuleti* and those on mice as well as those on mutant fruit flies recall the contradictions observed about the effect of berberine treatment on the consequent brain dopamine level in laboratory mammals, which was found to be increased in mice[20] and decreased in rats.[22]

As for cognition skills, honeybees with an artificially increased dopamine level were found to better learn and memorize odors.[24] Berberine did not change this faculty in *M. sabuleti* workers. Assessed on the linear and angular speeds, adaptation to the side effects of berberine did not occur, i.e., the ants did not recover from the negative effect of this product as long as they used it. A positive point is that berberine did not induce in the ants a significant dependence on its use, although a slight preference for it cannot be excluded. As explained in[54], there is no dependence on a product when its effect slowly decreases after weaning. Indeed, there was no ants' dependence on berberine, and the effect of this product, quantified by the ants' velocity, slowly decreased in 15 to 18 hours after the sugared solution of berberine was withdraw from the ants' diet. In addition, some weak dependence on this product cannot be ruled out as we observed a slightly faster decay of its effect at two time periods after its withdrawal. On the basis of our results on the action of berberine on the behavior of an ant used as a biological model, we believe that the study of the effect of this dietary complement on humans should include its action on behavior. For example, it should be ensured that berberine does not reduce the level of reactivity and vigilance, which could lead to dangerous situations when operating machinery or driving vehicles.

Conclusion

Few if no clinical studies in humans report about neurological and behavioral side effects of the alkaloid berberine. However, mild effects of this kind may exist. We thus made experiments for revealing such effects, using ants as biological models. We found that berberine present in the ants' sugar water diet in a proportion similar to the one used by humans, decreased their food appetence, motricity, and sensory perception, but not their state of stress, cognition, learning and memory. We suggest evaluating the effect of berberine on various associated behavioral and physiological traits in humans, for example their sensory perception and level of reactivity and alertness, especially as there are contradictions regarding the effects of this dietary complement on animals.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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