Prospecting for antimicrobial substances produced in four species of ants

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Abstract

Nowadays, we are witnessing the growth of difficult-to-treat infectious diseases caused by bacteria resistant to antibiotics. This resistance is most often caused by the misuse and improper use of antibiotics in both humans and animals. To remedy this problem, humans have turned to new sources of production of antibiotic molecules.

In this study, we focused on ants. It consisted in screening antimicrobial compounds from substances produced by ants and their microbiome. Four species of ants were used namely Paltothyreus tarsatus, Dorylus nigricans, Oecophylla longinoda, and Camponotus maculatus. First, successive extractions with three solvents (water, ethanol, dichloromethane) were carried out with the 4 species of ant. Only dichloromethane extracts from 3 ants, namely Dorylus nigricans, Oecophylla longinoda, and Camponotus maculatus, showed antimicrobial activity against Escherichia coli, Staphylococcus aureus and Candida albicans with minimum inhibitory concentrations (MIC) varying from 3 µg / ml to 15 µg / ml. Then, counting the microbiome of ants allowed 43 microorganisms to be isolated. Among them, two yeasts isolated from Camponotus maculatus showed antagonistic activity vis-à-vis Candida albicans.

these antimicrobial substances produced by these ants and their microbiota could be exploited as antibiotics or as precursors of antibiotics human and veterinary health

***Keyword**: Antibiotic Resistance, Ants, Microbiome, antagonistic activity and antimicrobial activity*

Introduction

One of the most significant therapeutic advances of the 20th century was the introduction of antibiotics after the Second World War. It is thanks to the work of Alexandre Fleming, author of the discovery of the first penicillin in 1928 and his collaborators Florey and Chain that this antibiotic could be used. Indeed, these new drugs have allowed a real medical advance. They have improved the prognosis of previously fatal bacterial infections and reduced the mortality caused by infectious diseases[1, 2].

However, with the overuse of antibiotics in human medicine, veterinary medicine and animal feed, bacteria have learned to defend themselves and adapt, and some have become resistant to antibiotics. The emergence of resistant bacteria is a public health problem that affects the hospital world, and, in a globalizing world, it is a problem that spares no continent. The increased mobility of individuals and that of foodstuffs constitute as many reservoirs favourable to the dissemination of resistant strains [3]. At this rate, WHO according to a report published in 2016 predicts that in a few years the world will move towards a post antibiotic era, where minor infections which have been treated for decades, could again kill [4].

Faced with the urgency of finding new therapies, preserving existing antibiotics and limiting the progression of resistance in the environment, new approaches are being developed. The first approach is to educate the public about the use of antibiotics, to set up systems for monitoring and following up on regulations concerning the use of antimicrobials. Another approach is to find other sources of production of molecules with antimicrobial powers. As an example, plants, some of which have been used by traditional medicine for centuries to fight infections [2].

Man could also draw inspiration from the animal kingdom, in particular insects. Social insects (bees, wasps, ants ...) that bring together tens of thousands of species, could also be an interesting way. They live in dense groups with a high probability of transmission of the disease and therefore have faced great pressure to develop defences against pathogens. Unlike wasps and bees, in which some species are social and others solitary, all free-living ant species are very social and should therefore invest in powerful antimicrobials.[5]. Ants are sensitive to a variety of fungal and bacterial pathogens and inhabit environments in which fungi and bacteria are abundant and diverse, such as soil and leaf litter [6-8].

It is with this in mind that our study focused on ants, thereby contributing to the quest for new antimicrobials. The main objective of this work is to screen for antimicrobial substances in ants. Its implementation calls for the evaluation of the antimicrobial activities of substances produced by the ant and the evaluation of the antagonistic activities of the ant microbiota against pathogens.

I. MATERIAL AND METHODS

A. Ant collections

Three species of ants (Paltothyreus tarsatus, Oecophylla longinoda, Camponotus maculatus and Dorylus nigricans) have been collected in the botanical garden of the University of Korhogo. The different species sampled colonize different types of soil and arboreal habitat. To collect ants in the field, forceps are used to catch them and the ants were introduced into sterile Falcon tubes. Three individuals of each species of ants were kept for their identification [9].

B. Extraction of substances produced by ants

The extraction method used was successive extraction with three solvents of different polarity: water, ethanol and dichloromethane. In our study, for each species of ants, 2g were first weighed in sterile Falcon tubes with a precision balance. Then 10 ml of sterile distilled water was added to it and stirred for 1 min, then, 24 h later, the mixture was filtered with Wattman paper. The residue obtained was used for two other extractions successively with ethanol and then dichloromethane under the same conditions as the first (Figure 1). The different filtrates obtained were freeze-dried (extraction with water and extraction with ethanol) or dried in an oven at 50 ° C (extraction with dichloromethane) in order to remove the solvents used for the different extractions. The aqueous extract (EAQ), the ethanolic extract (EET) and the dichloromethane extract (EDM) obtained are stored at 5° C protected from light before carrying out the antimicrobial tests [10].

The yield of the different extracts denotes the ratio of the mass of the extract after evaporation of the solvent by the initial mass of the ants. It is obtained according to the formula below.

Equation 1: Calculation of extraction yield

$$R = (m_f/m_i) \times 100$$

R: yield (%)
mi: initial mass of ant (mg)
mf: mass of the extract after evaporation of the solvent (mg)

C. Enumeration of the cultivable microbiome of ants

A quantity of ants was ground in a bench-top mortar, then 0.2 g of each species of crushed ants was weighed in an Eppendorf tube with the precision balance. Homogenization of each ground material is carried out in 1 ml of sterile distilled water to obtain the mother solution. Then a volume of 100 µl of the stock solution was added to 900 µl of sterile distilled water contained in an Eppendorf tube and then homogenized to obtain the 10^{-1} dilution. Four other decimal dilutions were made under the same conditions to obtain dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}.

To estimate the density of the microbial population, the surface inoculation method was used. For this, 100 µl of the various dilutions prepared were spread on the nutritive agar and Sabouraud agar then the petri dishes were incubated in the oven at 37 ° C and 30 ° C respectively for 24 hours and 48 hours. The colonies appearing on each petri dish are counted. The results are expressed in CFU/ml (Colony-forming Unit per milliliter) and given by the following relation:

Equation 2: Microbial enumeration in CFU/ml

$$N\ (UFC/ml) = \frac{\sum C}{V(n1 + 0{,}1n2)d}$$

V: Volume of inoculum (ml)
n1: Number of Petri dishes at the 1st lowest dilution at which the colonies are countable
n2: Number of dishes at the 2nd dilution following the first countable
d: Dilution retained
N: Number of colonies
CFU / ml: Colony-forming Unit per milliliter

The number of colonies was reduced to Colony-forming Units per gram.

Equation 3: Microbial enumeration in CFU/g

$$N' = N / C$$

With:
N': Number of colony forming units per gram of ant (CFU/g).
N: Number of colony forming units per ml of stock solution (CFU/ml).
C: Concentration of the stock solution in crushed ant

D. Antimicrobial activity test

a) Antagonist microbial activity test against pathogens

The aim here is to assess the antagonistic activity of isolated ant strains against pathogenic microorganisms. The spot method or also called the Fleming method used is a co-culture test on solid medium allowing to see the interactions between the isolated microorganisms and the selected pathogenic strains [11]. On the surface of a Mueller Hinton agar, 100 µl of a preculture of a strain of target pathogenic bacteria in the exponential growth phase were spread. The dishes were then dried at room temperature in a laminar flow hood for 15 minutes. At the end of this time, on the inoculated agar medium of each dish, 5 µl of each bacterial suspension of the microbiome of the

ants are delicately deposited in the form of a numbered spot. Each spot represents a bacterium of the ant microbiome. The petri dishes were incubated at 37 ° C for 24 or 48 h. The observation of an inhibition zone around the spot indicates the presence of antagonistic activity.

b) Determination of the minimum inhibitory concentration of ant extracts

The objective of this test is to assess the antimicrobial activity of the substances extracted in the various species of ants against pathogens. In a series of 6 sterile hemolyzed tubes, a concentration range of 0.5 ml of each ant extract is made by double dilutions with sterile distilled water. From a 24 hour incubation microbial culture, a preculture to reach the exponential phase of microbial growth is prepared for each target microorganism. Once its optical density at 600 nm has been obtained, this preculture is used to prepare an inoculum of 2.106 CFU / ml in 2 times concentrated Mueller Hinton broth. Then 0.5 ml of this inoculum are homogenized in each hemolysis tube of the range of concentrations previously prepared and then incubated at 37 ° C for 24 or 48 hours. At the end of this period, the turbidity of each tube is assessed with the naked eye in daylight and the smallest concentration in which an absence of turbidity represents the minimum inhibitory concentration of the compound tested on the microbial strain of the inoculum. used.

II. RESULTS

A. Substances extracted from different species of ants

The different extracts were obtained after extractions with three solvents: water, ethanol and dichloromethane. The extraction yields were determined from the ratio of the mass of the extract after evaporation of the solvent on the initial mass of the ants. According to our results (Figure 1), we note that the aqueous extracts have the highest yields (varies between 6.85% and 3.98%) in the four species of ants studied followed by dichloromethane extracts (varies between 2, 71% and 1.30%). Ethanol extracts vary between 1.81% and 0.78% and have the lowest yields.

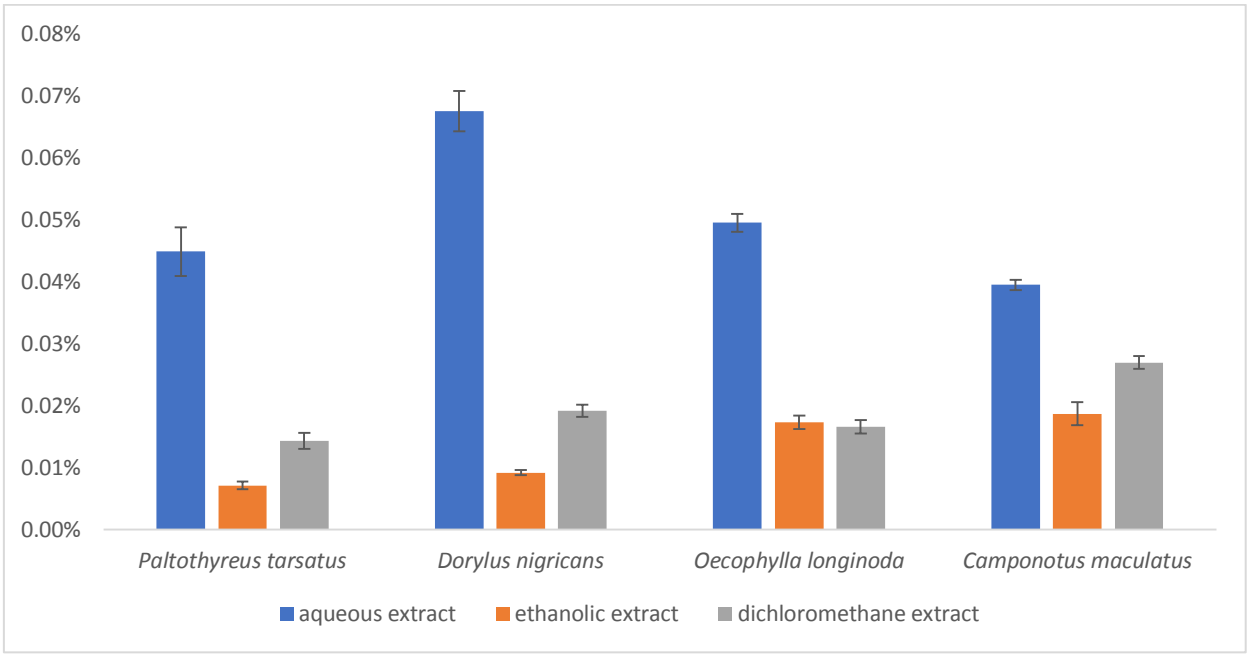

FIGURE 1; Yield of extracts from different species of Ants

B. Microbial strains isolated from ants

The count of the microbiome of ants is presented in FIG. 2. These results are presented in colony forming units per gram of ants. The ant species with the highest microbial density in the two culture media is *Dorylus nigricans* with a non-demanding total cultivable bacteria load of 1.16.108 CFU/g (on nutrient agar) and a cultivable fungal load of $8.71x10^7$ CFU / g (on Sabouraud). *Paltothyreus tarsatus* comes in second position with a bacterial density of 2.36 $x10^7$ CFU / g and a fungal density of $9.94.10^5$ CFU/g of ants body weight. Finally, *Camponotus maculatus* and *Oecophylla longinoda* have the smallest microbial loads with respective values of $2.57.10^6$ CFU/g and 3.64×10^5 CFU/g for their fungal density and 5.71×10^5 CFU/g then 2.36×10^6 CFU/g for their bacterial density.

Microscopic observation of the undemanding cultivable flora of the four ant species allowed us to note the absence of Gram-negative bacilli (BG-). The *Paltothyreus tarsatus* and *Oecophylla longinoda* species are dominated by cocci with respectively 57% and 33% for CG + then 43% and 50% for CG-. As for *Dorylus nigricans*, half of the bacteria are CG- and the other half are 25% CG + and 25% BG +. However,

Camponotus maculatus only presents Gram positive bacteria including 73% of CG + and 27% of BG + (Figure 3). The microscopic and macroscopic description of the bacteria and fungi isolated from the different ant species enabled us to distinguish 43 isolated microbial strains as shown in Table I.

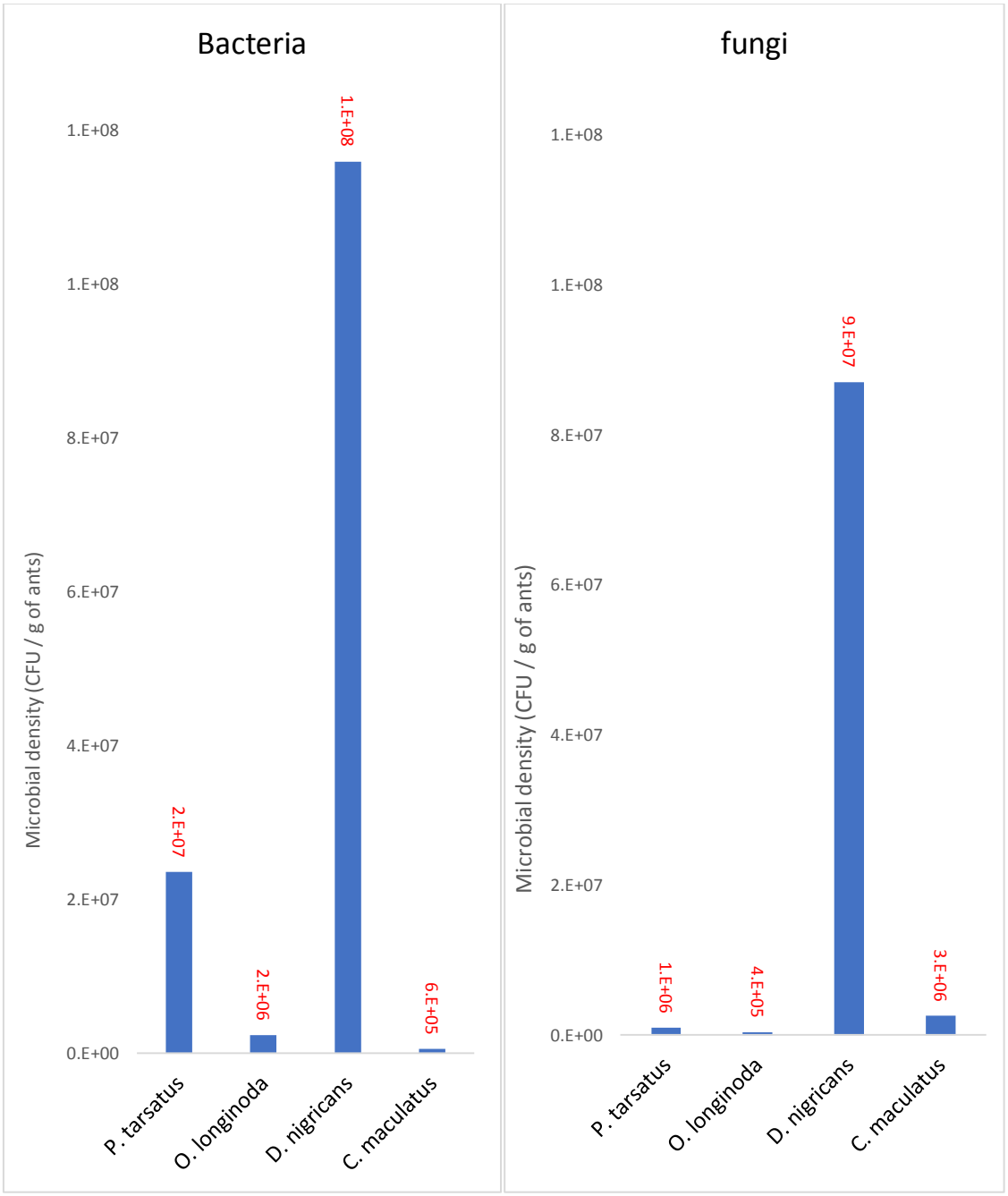

FIGURE 2: Enumeration of ants' microorganisms

TABLE I: distribution of microbial strains isolated in the four ant species

	Bacteria	fungi
Paltothyreus tarsatus	5	8
Oecophylla longinoda	3	5
Dorylus nigricans	6	7
Camponotus maculatus	6	3

C. Antimicrobial activity

The spot method was used to assess the antimicrobial activities of ants' microbiota against three pathogens (*Candida albicans, Escherichia coli and Staphylococcus aureus*). From the four species of ants, we were able to isolate 43 microorganisms including 20 bacterial and 23 fungal strains. No bacterial strain

has shown any antagonistic activity against the pathogens. However, there are two fungal strains which exhibit antimicrobial activity against *Candida albicans*. These two fungal strains codified FSs1 and FSs3 were isolated from the ant *Camponotus maculatus*. These 43 strains isolated from the four ant species showed no microbial activity towards *Escherichia coli* and *Staphylococcus aureus*.

The antimicrobial activity of the different extracts was carried out by the method of dilution in a liquid medium. This method allowed us to determine the minimum inhibitory concentration of the different ant extracts. The aqueous and ethanolic extracts of the different ant species showed no activity at the concentrations used in our tests. Only dichloromethane extracts from the four ant species (*Oecophylla longinoda, Camponotus maculatus and Dorylus nigricans*) showed antimicrobial activity against the pathogenic strains. The MICs of the various dichloromethane extracts from ants are shown in Table II. They range from 3 to 15 µg / ml. *Escherichia coli* presents itself as the bacteria most resistant to ant extracts while *Staphylococcus aureus* the most sensitive

TABLE II: MIC of dichloromethane extracts of different ant species

Ants	*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
Oecophylla longinoda	8.0 µg/ml	4.0 µg /ml	4.0 µg /ml
Camponotus maculatus	7.5 µg /ml	7.5 µg /ml	15.0 µg /ml
Dorylus nigricans	6.0 µg /ml	3.0 µg /ml	3.0 µg /ml

III. DISCUSSION

Ants are sociable insects; they live in society. This way of life can favor the spread of certain infectious diseases, but we rarely notice this case. So, we can deduce that the ants have a defense mechanism against infections. Our work aims to identify some ways in which ants can resist microbial infections. To achieve this, two hypotheses were put forward. The first is that ants could secrete antimicrobial substances themselves, which would make them less vulnerable to microbial infections. The second is that ants could harbor microbiota, microorganisms that protect them from pathogenic microorganisms. These are microorganisms which exhibit antagonistic activities against pathogenic microorganisms of ants.

This work allowed us to identify the probable sources of protection of four species of ants (*Paltothyreus tarsatus, Dorylus nigricans, Oecophylla longinoda, Camponotus maculatus*) against certain pathogenic microorganisms. Thus, to demonstrate the first hypothesis, we carried out extractions with three different solvents (water, 70% ethanol and dichloromethane) on the four species of ants. The use of solvents with different polarities has made it possible to separate the compounds from the ant according to their degree of solubility in the extraction solvents. The different yields reveal that the ants studied in this work are rich in polar compound, since the aqueous extracts have the highest yields.

The antimicrobial activities of the twelve extracts obtained (4 aqueous extract, 4 ethanolic extract and 4 dichloromethane extract) were evaluated against *Escherichia coli* (Gram negative bacteria), *Staphylococcus aureus* (Gram positive bacteria) and *Candida albicans* (yeast). The results of these tests revealed that only dichloromethane extracts have antimicrobial activity against target microorganisms. The minimum inhibitory concentrations (MIC) vary from 3µg / ml to 15µg / ml. It appears that the dichloromethane extract of the ant *Dorylus nigricans* has the best antimicrobial activity against the three target microorganisms. Also, we note that Staphylococcus aureus presents itself as the most sensitive microorganism, however Escherichia coli presents the smallest sensitivity. Thus, we can say that three of the four species of ants studied (*Oecophylla longinoda, Camponotus maculatus* and *Dorylus nigricans*) possess antimicrobial substances. The results obtained are consistent with the work of Penick, Halawani [12] where the ethanolic ant extracts had antibacterial activities against Staphylococcus epidermidis. Beattie, Turnbull [13] showed that the secretions of the metapleural gland of the Australian ant *Myrmecia nigriscapa* significantly reduced the germination of spores of species of pathogenic fungi of ants. These antimicrobial compounds would be secreted by an organ of the ant in the event of infection or in the presence of pathogens in its environment. With regard to our second hypothesis, the enumeration of the microbiome of the four species of ants was carried out on the nutritive agar (GN) to isolate the total non-demanding cultivable bacterial flora and on the Sabouraud agar (SAB) to count the fungi. The variable microbial density observed in the different species of ants studied comes from their environment, their way of life and their diet. *Dorylus nigricans* has the highest microbial density among the ant species in this study. This could be justified by their nomadic life.

The description of bacteria from non-demanding total flora shows a dominance of gram negative and positive cocci. Whatever the ant species studied; we noticed an absence of gram-negative bacilli. This absence of

gram-negative bacilli among isolated bacteria was also observed by Birer [9], who worked on the bacterioma of 15 species of ants.

Tests of antimicrobial activities by the Fleming method were carried out to identify among the microbiota of ants, those which would have an antagonistic activity against the three pathogenic strains. This is a non-quantitative test to just identify the microorganisms of interest. The results indicate an antifungal activity of two fungal strains isolated from *Camponotus maculatus*. This observation shows that the ant lives in symbiosis with these fungi which would help it fight against certain microbial infections. This type of observation has been reported by Currie, Scott [14] and Ishak, Miller [15]. Research by the latter has highlighted the antifungal activities of certain bacteria living with ants. This observation was also made by Birer [9] who, from 15 species of ants, identified 22 bacteria out of 43 isolated which show antagonistic activities against *Staphylococcus aureus, Escherichia coli, Candida albicans, Enterococcus faecalis, Pseudomonas aeruginosa and Beauveria bassiana* .

This type of association (host-microbiota) has also been observed in animals, insects and even plants [16-18] while highlighting the <protective role of microbiota on the host. The results thus observed show us that the Camponotus maculatus ant has microorganisms which could help it to fight against pathogenic microorganisms by protecting it from microbial infections. These microorganisms are said to secrete bioactive substances against pathogens [16]. This relationship between the host (the ant) and the microbiota is so important that one could speak of an interdependence between these two entities. This observation has led some authors to consider the host and the microbiota as a single evolutionary entity. This evolutionary entity made up of a network of biological molecules of the host and its associated microorganisms, is called "holobionte" [19-21].

Conclusion and perspectives

To slow the spread of pathogenic microorganisms resistant to antibiotics which is a major public health problem, a quest for new antibiotic molecules is necessary. It is in this sense that this work, which consists in identifying antimicrobial substances in the ant, has been oriented.

This study was first oriented towards the substances produced by the ants through their extracts with different solvents. Then we were interested in the antagonistic activities of the microbiota of ants against pathogenic microorganisms.

On the one hand, we have shown that the dichloromethane extracts of three of the four species of ants showed antimicrobial activities. The MICs of these extracts vary from 3µg / ml to 15µg / ml against *Escherichia coli, Staphylococcus aureus* and *Candida albicans.* On the other hand, two strains of fungi from the species Camponotus maculatus have shown antagonistic activities against *Candida albicans.*

At the end of this study, we will retain that ants have a system of protection against microbial infections. this system comes from the antimicrobial substances they secrete and also from the beneficial effects of their microbiome. Thus, the antimicrobial substances they produce could be used as antibiotics or antibiotic precursors in the treatment of infectious diseases in human and veterinary health.

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