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Secondary metabolites isolated from the bacterial complex of *Apismellifera* and their action against *Atta mexicana*

Rivera A[1], Romero O[2*], Hernandez F[3], Badillo F[4] and Aragon A[2]

[1]*Centro de Investigaciones en Ciencias Microbiologicas, Instituto de Ciencias de la Benemérita Universidad Autonoma de Puebla, Mexico.*
[2]*Centro de Agroecologia del Instituto de Ciencias de la Benemérita Universidad Autónoma de Puebla, Mexico.*
[3]*Centro de Química del Instituto de Ciencias de la Benemerita Universidad Autonoma de Puebla, Mexico.*
[4]*Posgrado en Manejo Sostenible de Agroecosistemas, Instituto de Ciencias de la Benemerita Universidad Autonoma de Puebla, Mexico.*
Correspondence E-mail: biol.ora@hotmail.com

ABSTRACT

Damage caused by insects in agriculture has led to a significant decrease in crop quality, yield and economic losses. So this situation has led to the indiscriminate use of synthetic chemicals that have sought to mitigate negative impacts on the crops, but without considering the pressure and imbalances that are causing on ecosystems. Therefore it is necessary to direct research into new biotechnological alternatives, focused on the control of pests which lead to the decrease of the acquisition of external inputs in agroecosystems, framing production systems in sustainable agriculture that produces healthy, sufficient food and quality cost-effective, socially acceptable and without harming the environment. We evaluated the ability of metabolites produced by bacterias isolated from Apis mellifera as insecticides against Atta mexicana. Metabolites extraction was carrying out from the Enterobactericiae isolated, Klebsiella, Pseudomonas, Proteus and Yersinia, by using an adsorbent resin and elution with 99% ethanol. The insecticidal activity of metabolites was tested in vitro using experimental units against Atta mexicana. Yersinia metabolites showed insecticidal activity over Atta mexicana showing an accumulative mortality of 92.5%. Protein profile was performed on the metabolites, using dodecyl sulphate-polyacrylamide gel, where variability was observed in weight and number of protein bands, predominantly 70 kDa. Yersinia showed protein bands of 10 and 70 kDa, the weight could be comparable to Cry endotoxins of Bacillus thuringensis. Results suggest that Atta mexicana can be considered as a promissory source of by bacterias with varying activity that could be used as alternatives biotechnological protection crops against Atta mexicana.

Key words: Bacterial complex, secondary metabolites, insecticidal activity, *Apismellifera*, *Atta Mexicana*.

INTRODUCTION

All agriculture before the development of synthetic pesticides, can be considered as a biological agriculture, but as it progresses and knowledge systems are modernized agricultural production, ecological principles are easily ignored, causing unstable agricultural systems; this instability is reflected in the rise of agricultural pestscausing significant yield reductions and economic losses worldwide[1]. In Mexico are frequent and significant damage caused by pests in crop plants and stored agricultural products. Recent studies show that losses caused by ants of the genus Atta, better known as leaf cutter ants are considered as one of the most important agricultural pest in neotropical regions [2].

Faced with this problemhas becomeindiscriminate useof synthetic pesticides, this being a symptom of theenvironmentalcrisis affectingagriculture.However, the indirect cost ofpesticidesfor harmto the environment

andpublic health mustbe balanced againstthese benefits.So in recent years the trend towards finding a sustainable agriculture and the growing public concern about the risks associated with synthetic pesticides, has resulted in technologies that include the use of natural or modified organisms, their genes, the product their genes [3],as well as bioactive compounds (secondary metabolites); product of these organisms which reduce the undesirable effect on plants or animals, representing, biotechnology alternatives [4],that may be more effective, biodegradable and less toxic to non-target organisms [5-8].

In this context, it can be considered to bee*Apismellifera* as a potential source of bioactive compounds in your body to hold a high bacterial load capable of producing secondary metabolites with diverse activities [9], in response to the natural competitiveness observed in the different environments in which the incidence has pollinator [10], as well asinteractionswith other populations, the bacteriabeingable to producesuch compoundsnot onlyfor self-defense, but also tothat ofits host[11]. In this regard, given the great diversity of bacteria from various sources, it is of interest in the field of sustainable agriculture, identification and characterization of these bacteria as biocontrol agents, by producing their bioactive compounds[12].

The ant genus Atta, are commonly known as leaf-cutter ants, ants, forage or cut sheets; they differ from other kinds of ants that cut Attini plant material for growing fungus *Leucocoprinus*sp, which is their main food and which developed a symbiotic relationship. *Atta mexicana* causing great losses in the tropics to defoliate sesame, cotton, peanuts, coffee tree, onion, chili, citrus (lime, lemon, tangerine), beans, jamaica, corn, papaya, banana, wheat, vine, yucca and ornamental plants[13, 14], this species is distributed from Arizona, Mexico, Guatemala, Belize, El Salvador, Honduras and Nicaragua[15, 16]. Although the work of these insects is ecologically important because they remove soil, promote ventilation, provide organic matter and improve soil structure, high impact as does trimmers and unwieldy to be considered as an important pest in systems agricultural and pasture in several countries in Latin America[16, 17],however studies show that although the losses caused by this pest are enormous, do not have systematic records by country or region[18]. The aim of this study was to evaluate the activity produced by bacteria isolated from *Apismellifera* against *Atta mexicana*secondary metabolites.

MATERIALS AND METHODS

Specimens of *Apismellifera* were collected from apiaries located in Santa Maria Coronango, Puebla-Mexico. This community is located between parallels 19° 06 'and 19° 10' north latitude; meridians 98° 15 'and 98° 20' west longitude; altitude between 2180 and 2200 m.The climate is temperate humid with summer rains. It has a temperature range between 14°C and 18°C and rainfall ranging between 800-1000 mm.

Sampling was conducted from January 13 to May 12, 2014 randomly in the apiary consisting of 30 hives, taking 10% of all cases present for weekly sampling, under the following procedure, the hives were located sampling, with the help of a smoker box opened and a total of 30 bees were taken for each sample.They were made with sterile forceps and also stored in a sterile bottle labeling. They were transported as quickly as possible to keep the form of live specimens the laboratory for processing.

The isolation of bacterial colonies was performed by processing seven live specimens of *A. mellifera*, which were placed for one minute at a temperature of 4°C, this in order to decrease their living functions and process. Then suspended in 10 ml of nutrient broth and macerated with a mortar, made aliquots in separate tubes and incubated at 30°C for 24 hours, then tenfold dilutions were made from this sample and replated on nutrient agar by groove 20 µl of each dilution and incubated at 30°C, 24 hours. Of total colonies they were isolated a total of 17 morphologically different strains.

The 17 isolates were identified by the gallery systemAPI 20E^{TM} (20 100/20 160 BIOMERIUX).Every 17 isolates were inoculated into 5 mL of nutrient broth, achieving a strain suspension, which was incubated for 18 hours at 30°C. After this period took 20 mL of the bacterial suspension and inoculated into each of 20 wells.

Secondary metabolites were produced in triplicate, inoculating a colony of each bacterial genus isolated in 5 mL of nutrient broth and incubated at 37°C overnight. The next day 500 µl of this culture was placed in a 125 ml flask with 50 mL of nutrient broth.Simultaneously, they added 2 mL (4% w/v) adsorptive resin Amberlite XAD16 (Sigma), in aqueous solution. Incubation was performed at room temperature for 5 days at 180 rpm.Upon completion five days of incubation, the resin was recovered by decantation and attached to it metabolites were eluted with 20 mL of 99.9% ethanol and concentrated with the aid of a rotary evaporator to obtain a milliliter of extract. The concentrates were stored at -20°C,two concentrations of each were obtained 15% and 7.5%[19].

For in vitro evaluation of the activity of the extracts against *Atta mexicana* army ants specimens of which were obtained from the anthill taken into captivity in the Entomology Laboratory of Agroecology Center, Benemerita Universidad Autonoma de Puebla.

The insecticidal activity of extracts was evaluated considering the susceptibility of the ant, the contact with the extracts. Seven treatments were tested, considering four extracts at a concentration of 15%, treatment with 70% ethanol, a positive control using *Aspergillus* sp. and target (untreated).

Four replications were made, and tested under a completely randomized design, so he had a total of 28 experimental units, the experimental unit was a Petri dish with 20 ants with food (0.15 g mushroom *Leucocoprinus*sp.). 285 µl of the respective treatment, same as sprayed on ants and maintained in a breeding chamber of insects temperature of 26 ± 2°C, relative humidity of 70 ± 10% and photoperiod of 12 hours light were applied, to be monitored the mortality of individuals at 24, 48, 72, 96, 120, 144 and 168 hours.

Once results were obtained as to the insecticidal activity by one of extracts from the genus *Yersinia* secondary metabolites, a second experiment, where 5 treatments tested were established; extract secondary metabolites of *Yersinia* at two concentrations (15 and 7.5%), treatment with 70% ethanol, one using *Aspergillus* sp control and target (untreated).

Four replications were conducted and tested under a completely randomized design with 4 repetitions, so he had a total of 20 experimental units, where the experimental unit was a micro anthill with 20 ants with food (0.15 g mushroom *Leucocoprinus*sp) and peach tree leaves. 285 µl of the respective treatment, same as sprayed on ants were applied, and mortality of individuals at 24, 48, 72, 96, 120, 144 and 168 hours was monitored.

The data obtained were evaluated with analysis of variance (ANOVA) and respective Tukey test, with a confidence level of 95%, using the program Statgraphics Centurion XVI.

RESULTS AND DISCUSSION

Apismellifera bee showed microbiota consists of four bacterial genera, with predominance of *Klebsiella* and *Pseudomonas*, followed by the genera *Yersinia* and *Proteus* (Table 1). These results agrees with those reported by Gilliam (1987), Gilliam *et al*., (1988) and Gilliam and Taber (1991) whose works relate bacteria to the family Enterobacteriaceae, are the most numerous, mainly present in the intestinal tract microorganisms bee [20-22].

White (1921), cites the microbiota of *Apismellifera*, which reports the presence of *Lactobacillus rigidusapis*.,*Lactobacillus constellatus* and *Bacillus influzoidesapis*. Martinson *et al*. (2012) reports that the bacterial load that has bee mainly housed in your gut and thanks to the sociability of the species and according to Dillon and Dillon (2004), the distinguishing bacterial flora bee is transmitted may play a role health and vitality of these organisms [23-25].

Because of the importance of *Apismellifera* and their relevant contribution to agro-ecosystems as a natural pollinator [26, 27], is that the recent losses of colonies have captured the public interest, especially in countries like the US and Europe, with the places where the highest mortality rates are reported, with averages ranging 30% [28, 29], these reports also suggest that the reasons this disorder vary by region or country and cannot be attributed to one factor, so that efforts to reduce losses should be different [30]. Faced with this problem some authors mention that the greatest loss may be attributable to the introduction of pests and pathogens, which suggests the need to study and characterize bacterial biota of the bee as a strategy of good management and conservation of the species [31, 32], as many of the identified bodies are not only related to digestion and absorption of nutrients, but with pathogenic effects on their hosts [33].

Microbiota isolated in specimens of *A. mellifera*, 4 different extracts, which correspond to secondary metabolites of *Pseudomonas*, *Proteus*, *Klebsiella* and *Yersinia* were obtained. Concentrate each sample dilutions were made in distilled water and two final concentrations 15% and 7.5%were obtained.

The insecticidal activity obtained with the different extracts was evaluated from the percentage of mortality, where the analysis of variance with their respective Tukey test (Table 2) allowed seen from 48 hours significant differences (p value <0.05) the effect of the other treatments groups were 3 medium, where the treatment was *Yersinia* which cause a higher mortality 21.25% while *Proteus* treatment was the treatment cause lower mortality (1.25%). The analysis also allowed observing the prevalence of this trend throughout all measurements, totaling *Yersinia* mortality by 92.5%.

At 168 hours shown in Table 2, four groups of medium being based treatment *Yersinia* which had the highest mortality, and is statistically different (p value <0.05) to the effect of other treatments, as can be seen white treatment showed high mortality, this may be because the ants can not survive long without the queen.

The results obtained in this research related to the insecticidal activity of secondary metabolites of the genus *Yersinia*, can be explained, as there are reports where found *Yersinia entomophaga*showing no toxicity to humans, but for insects [34, 35]. According to Hurst (2011) *Yersinia entomophaga* MH96 bacteria, it has the ability to cause death through a wide range of insect species, including the order Coleoptera and Lepidoptera [36]. Ffrench-Constant *et al*., (2007) mention that the insecticidal activity of *Yersinisentomophaga* is determined by the presence of a complex of toxins according to Blackburn *et al*. (2011), this complex is found in *Bacillus thuringiensis*, known bacterium used successfully in controlling crop pests [37, 38]. However the recent emergence of resistant insect toxins of *B. thuringiensis* (Tabashnik*et al*., 1993)is one of the main reasons that have motivated the search for new sources of biopesticides, such as *Yersinia* [39].

The determination of the protein profile was performed because it was considered that agents causing the toxicity could have protein origin, so for determining electrophoresis was used in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE), since it is considered as an effective method for the differentiation of microorganisms [40] as well as being a reliable technique and commonly used for the separation, identification and characterization of proteins [41, 42].

The determination of protein profiles to each of the extracts (*Klebsiella*, *Pseudomonas*, *Proteus* and *Yersinia*) isolated from *A. mellifera* biota, variability were observed in the number of protein bands, but showed similarity in their size, predominant molecular weight of 70 kDa, which was observed in all profiles. The band of molecular weight of 10 kDa coincided with *Yersinia* and *Pseudomonas*, while molecular weight bands 55, 70 and 130 kDa, agreed to *Pseudomonas* and *Proteus*, the latter extract which had the highest number of bands (Figure 1).

Toxicity of the four extracts obtained from strains identified was evaluated, where only the extract from the genus *Yersinia* showed insecticidal activity against *Atta mexicana*, whose protein bands corresponding to a molecular weight of 10 and 70 kDa. This fact can be attributed to the presence of toxic proteins by molecular weight could be compared to some of the endotoxins of *Bacillus thuringiensis* Cry because according to the literature it is known that the insecticidal activity that is attributed to the bacterium *Bacillus thuringiensis* is due to its ability to produce a parasporal body proteinaceous known as crystal during sporulation phase. This protein crystal endotoxin is known as Cry or Cyt proteins, some of which are active against insects such as Lepidoptera (butterflies), Coleoptera (beetles), Diptera (mosquitoes), mites and hymenoptera (ants), among others.The molecular weight of the Cry proteins between 25 and 140 kDa[43, 44]. Currently 332 endotoxins have been classified into 47 groups of Cry proteins, 22 proteins Cyt into two groups and six other proteins of *Bacillus thuringiensis* that have not been classified in a specific group [45]. Cry toxins to one toxic fragments weigh between 60 and 70 kDa, for Cry 2 the molecular weight is 70 kDaprototoxin, like for Cry 3, however they differ in the type of crystal formation being for Cry 2 cuboidal and diamond for the Cry Cry 3. 4 have found that the toxic fragments correspond to molecular weights ranging from 50 to 70 kDa[46]. 70 kDa polypeptides which correspond with endotoxin Cry type 2 associated with cubic morphology are active both Lepidoptera and Diptera[47, 48] but so far, with the except for a patent application of toxin-specific ant Cry22, Cry protein whatsoever has been found to be directly toxic to insects Hymenoptera. According to Soberon and Bravo (2007), the mode of action of Cry toxins are observed by symptoms that occur from ingestion of crystals, such as cessation of intake, paralysis of the bowel, diarrhea, total paralysis and ultimately death [49]. Generally Cry toxins are lytic pores exert their toxic activity to cause osmotic imbalance in the epithelial cells which are inserted into the membrane [50]. Therefore although this may explain the possible relationship between the proteins found in the extract of *Yersinia* with molecular weight of 70 kDa and toxicity against *Atta mexicana*contact the endotoxins of *B. thuringiensis* Cry, and they have now found Cry toxins in other bacterial species *Clostridium bifermentants*can not be assured that it is the same as it has not been proven the similarity in their amino acid sequences in this way can be explained lack of insecticidal activity in the other extracts also showed bands with molecular weights of 70 kDa [49].

Table 1: Bacterial genera isolated from *Apismellifera*

Gender	%
Klebsiella	37.50
Pseudomona	31.25
Yersinia	12.50
Proteus	6.25

The insecticidal activity exhibited by the extract of *Yersinia* on *Atta mexicana*can respond to the need to implement new biotechnology alternatives in combating pestsencuadren with agroecological development, replacing the

intensive and uncontrolled use of pesticides, causing the generation of resistance already well-studied and damage to the environment and human health.

Table 2: Mortalityassessments

	Mortalities percentage±Error Standart + Significance						
Tx	24hours	48hours	72hours	96hours	120hours	144hours	168hours
E_1	2.5±1.44 a	7. 5±1.44 b	8.75 ± 1.25 b	27.5 ± 1.44 b	37.5 ± 1.44 b	43.75 ±1.25 b	65 ± 0 c
E_2	1.25± 1.25 a	5± 0 b	8.75 ± 1.25 b	12.5 ± 1.44 c	20 ± 2.04 c	40 ± 2.04 b	71.25±1.25 b
E_3	2.5±1.44 a	21.25±1.25 a	31.25 ±1.25 a	47.5 ± 1.44 a	73.75 ±1.25 a	76.25 ±1.25 a	92.5 ± 1.44 a
E_4	1.25±1.25 a	1.25±1.25 c	1.25 ± 1.25 c	3.75 ± 1.25 d	12.5 ± 1.25 c	30 ± 0 c	52.5 ±1.44 d
C+	1.25±1.25 a	6.25 ±1.25 b	10 ± 0 b	12.5 ± 1.44 c	13.75 ±1.25 c	31.25 ±1.25 c	53.75±1.25 d
C-	2.5±2.5 a	6.25±1.25 b	8.75 ± 1.25 b	13.75 ±1.25 c	28.75±1.25 b	42.5 ± 1.44 b	63.75±1.25 c
B	0±0 a	3.75±1.25 b	7.5 ± 1.44 b	15 ± 0 c	32.5 ± 1.44 b	45 ± 0 b	70 ± 0 b

Tx: Treatments, E_1: Pseudomonas, E_2: KlebsiellaE$_3$: Yersinia, E_4: Proteus, C+:Aspergilus, C-: Ethanol, B: Target.
**Different letters in the same column indicate significant difference, Tukey test (α = 0.05).*

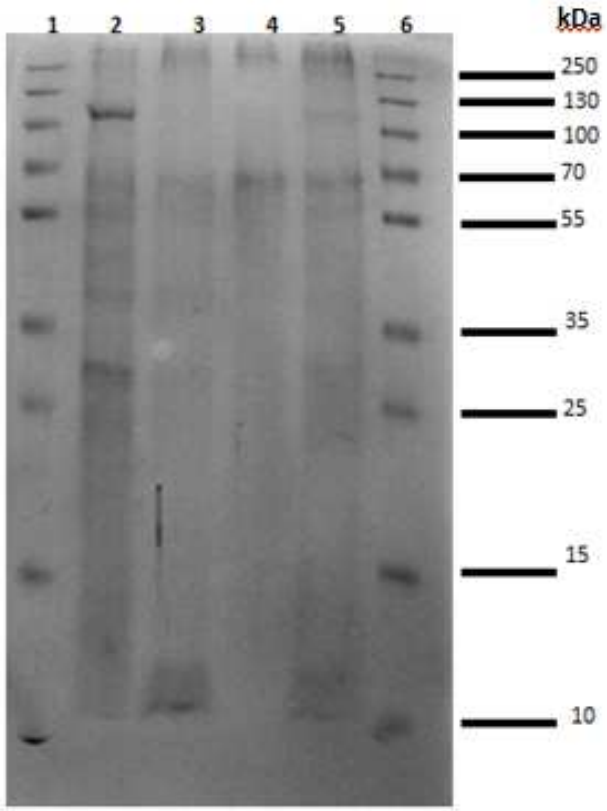

Fig. 1. SDS-PAGE 10% extracts of *Proteus* culture (lane 2), *Yersinia* (lane 3), *Klebsiella* (lane 4), *Pseudomonas* (lane 5), Lane 1 and 6 molecular weight marker.

CONCLUSION

Were isolated and identified four bacterial types: *Klebsiella* (37.5%), *Pseudomonas* (31.2%), *Yersinia* (12.5%) and *Proteus* (6.2%). The secondary metabolites produced by *Yersinia* presented proteins with molecular weights of 10 kDa and 70 kDa.It was possible to observe that the Yersinia extract contains biologically active secondary metabolites against *Atta mexicana*by exhibiting a 92.5% cumulative mortality.

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